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Some cellulose ion exchangers of low substitution and their chromatographic application

By Jerker Porath

With 3 figures in the text

By means of chromatography or a combined effect of electrophoresis and chromatography, Lautsch *et al.* (1) separated amino acids on "ion exchange paper". The first successful fractionations of natural peptide materials on a preparative scale with cellulose derivatives was performed by Astwood *et al.* (2). Most of the ascorbic acid depletion factor from ACTH preparations of low activity could be adsorbed on oxycellulose in weak acetic acid solutions and subsequently desorbed by lowering the pH. It is, however, due to Sober, Peterson, and their collaborators (3, 4) that attention has been drawn to the suitability of ionic cellulose derivatives as adsorbents for proteins and nucleic acids.

Each glucose unit of cellulose contains one primary and two secondary hydroxyl groups. As a rule, the hydrogen of these hydroxyl groups is largely substituted in the case of commercially manufactured derivatives of cellulose. Consequently the cellulose products differ distinctly from each other and from native cellulose both with respect to chemical and physical properties. However, occasionally, in the textile industry, it is desirable to introduce reactive groups while still retaining the structure and strength of the original fiber. Some slightly substituted derivatives have better dyeing properties than native cellulose; thus, the introduction of basic groups makes for faster dyeing with acid dyestuffs, etc. All products of this type having a low solubility and only a slight tendency to swell are potential adsorbents for ion exchange chromatography.

The cellulose derivatives which have hitherto been used have either been ethers or esters with the exception of oxycellulose. The ethers are as a rule more stable in strongly acid and basic solutions than the esters, making a more complete regeneration of the former type of ionic exchangers possible.

A chromatographically suitable product of the best known ionic cellulose ether, carboxymethyl cellulose ("CM-cellulose"), has been prepared by Sober and Peterson (3).

The author is unaware that powder or pulp for chromatographic use containing other acid groups coupled to the cellulose skeleton via ether bonds has as yet been prepared. The synthesis of sulfomethyl and sulfoethyl cellulose will be described below. Of these, sulfomethyl cellulose appears to be a new cellulose derivative, while sulfoethyl cellulose has been prepared earlier by Karrer *et al.* (5), Timell (6) and others. The extensive use at present of sulfonic acid ion exchangers for the preparation and analysis of natural products, not the least of which are amino acids, makes

cellulose ion exchangers with alifatically bound sulfonate groups of special interest as a complement to sulfonate ion-exchange resins.

The diethylaminoethyl ether of cellulose was prepared first by Hartmann (7), and subsequently by Hoffpauir and Guthrie (8), by heating a mixture of β-chloroethyldiethylamine with alkaline cellulose. Using this method Sober and Peterson have prepared ion exchangers in powder form ("DEAE-cellulose" (3)), and have demonstrated their suitability for protein chromatography in a convincing manner. Starting from DEAE-cellulose the author has prepared a quaternary ammonium base, triethylaminoethyl cellulose (TEAE-cellulose) by ethylation. A similar product has also been prepared by Hoffpauir and Guthrie (8).

Experimental

A. Preparations and analyses

Sulfomethyl cellulose (SM-cellulose).

Sulfomethyl cellulose has been synthesized using sodium chloromethane sulfonate or the corresponding bromine compound. The former substance is preferable because of its greater reactivity. Therefore, only the synthesis based on it will be described.

Sodium monochloromethane sulfonate.—2 moles of dichloromethane, dissolved in 200 ml ethanol, are mixed with 1 mole of sodium sulfite, dissolved in 300 ml water, in a high pressure autoclave provided with a stirrer. The mixture is heated to 120°C. After 6 hours, with continuous stirring the heating is discontinued and the reaction mixture is transferred to a large porcelain dish and concentrated to dryness on a water bath. The orange colored residue is pulverised in a mortar and then subjected to continuous extraction with boiling methanol. Approximately 70 grams of crystals can be collected from 100 ml of methanol after cooling. An additional 35–50 grams can be obtained by working up the mother liquor. The crystalline material is pulverized and dried by suction on a Büchner funnel. This crude product can easily be purified by recrystallization from methanol.

The infrared spectrum shows a strong, well defined absorption band with a maximum at 8.3 μ and a somewhat weaker band at 9.4 μ. The existence of these bands indicates the presence of sulfonate groups. As may be expected of a C–Cl bond, a strong maximum occurs at 13.9 μ (9). The crude product contains more than 95 % monochloromethane sulfonate as found by sulfur and chlorine analyses.

The conditions for the synthesis of the sulfomethyl ion exchanger have been varied in many ways. Thus, different relative amounts of alkali, cellulose, and chlorosulfonate have been employed. Further, the temperature and reaction time have been studied. The reaction between the alkali-cellulose and the chloromethane sulfonate is considerably slower than the corresponding reaction with monochloroacetate. A higher temperature and a lower water content are necessary for satisfactory reaction. Some products have been synthesized under nitrogen atmosphere, but the exclusion of oxygen is usually not necessary for the production of chromatographically satisfactory ion exchangers. When even the presence of small amounts of carboxylic groups is deleterious, all operations involving alkali-cellulose should be carried out in the absence of oxygen.

The following simple but dependable method has been used routinely.

100 g of shredded cellulose linters or Munktell cellulose powder is mixed with a solution of 200 g of sodium hydroxide in 300 ml of water. The mixture is subjected

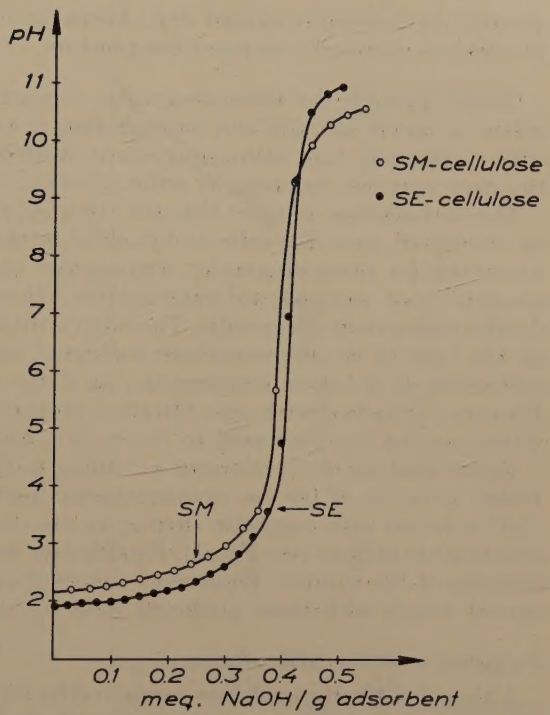

Fig. 1. Titration curves of SM and SE-cellulose.

to effective stirring with a strong mechanical mixer and allowed to swell for 4–12 hours. 10 g of crude sodium chloromethane sulfonate dissolved in 60 ml of water are now added in portions with thorough mixing. The cellulose mass is transferred to a weighed glass or metal dish and placed in an oven at 90–95° without attempting to prevent evaporation. (A more uniform product should be obtained with vacuum concentration in a rotating drum. This also prevents oxidation.) The mass is stirred occasionally and weighed. When 80 per cent of the water added to the cellulose had evaporated the reaction is discontinued. A sample is cooled, suspended in water, decanted, neutralized, and sucked dry on a Büchner funnel. It is subsequently washed with 0.5 *M* hydrochloric acid, then with water until the reaction is neutral, with ethanol, and finally with ether. After the ether has been removed by evacuation, the product is weighed, dissolved in 0.5 *M* aqueous NaCl solution, and titrated with 0.1 *M* NaOH. The cellulose should be substituted with at least 0.35 meq. sulfonate groups per gram of dry powder. If the sulfonate content is unsatisfactory, heating of the main part of the reaction mixture is continued until the water content has decreased to 14 %. The entire reaction mixture is now cooled and poured out into 1 liter of 95 % ethanol. One liter of 1 *M* HCl is slowly added with stirring and cooling. When the heat liberation has decreased, stirring is discontinued and the mass allowed to sediment. One liter of fresh 1 *M* HCl in ethanol is added and the procedure is repeated. After a second sedimentation the cellulose ion exchanger is transferred to a large Büchner funnel and the solution is removed by suction. After washing with 1 liter of 0,5 *M* HCl and subsequently with water to neutral reaction, the white

product is thoroughly sucked dry. A sample for analysis is dried in vacuum over phosphorus pentoxide at room temperature.

Before packing the chromatographic column, the ion exchanger is suspended in water or buffer solution and homogenized if necessary in a mixer. Ion exchangers with a relatively high sulfonate content must be suspended in a larger volume and the finer particles removed by sedimentation.

The introduction of more than 0.5–0.6 meq. sulfonate per g of cellulose should not be attempted, since the sulfomethyl cellulose then tends to become gel-like and thus unsuitable for chromatography. Sulfomethyl cellulose preparations with more than about 0.7 meq. per gram are water soluble. When a solution of this type of material is dried a transparent film results. The sulfomethylation yield is approximately 40–50 % on the basis of the chloromethane sulfonate, but can be increased if the reaction is performed at a higher temperature, or if the water content is further decreased. However, a product with poor filtration properties has often been obtained when the water content has decreased to below 25 % and the temperature exceeds 100°C.

Sulfur analysis was performed according to Ottoson and Snellman (10). Potentiometric titration of the ion exchangers was performed with 0.1 *M* NaOH in 0.5 *M* NaCl solution with magnetic stirring as described by Peterson and Sober for other cellulose exchangers (see Fig. 1). Equilibrium was attained a minute or so after each addition of the titrator. Equivalent values of acidic groups as obtained by titration agreed closely with those predicted from the sulfur content.

Sulfoethyl cellulose (*SE cellulose*)

Although sulfoethyl cellulose seems to offer no advantage over sulfomethyl cellulose, the author has decided to publish a method of synthesis of a sulfoethyl cellulose product which has been used by others at this institute for protein fractionation.

Alkali cellulose is prepared by soaking 100 g of shredded cellulose linters with 300 ml of alkaline solution, obtained by dissolving 300 g of sodium hydroxide in 600 ml of water. The mass is thoroughly stirred with a mechanical mixer and allowed to swell for 24 hours at room temperature. To 120 g of this alkali cellulose is added in small portions 30 g of sodium bromosulfonate (11) dissolved in 20 ml of water. The mass is thoroughly stirred after each addition and finally heated to 120° in nitrogen atmosphere. This temperature is retained for 3 hours, during which the mass is occasionally stirred. The light yellow product is then allowed to cool to room temperature, after which it is mixed with 500 ml of ethanol in a mixer. The product is sucked dry, suspended in 4 liters of water, and the suspension is acidified with 5 % H_2SO_4. When the main part of the cellulose ether has sedimented, the supernatant is decanted. Then the sedimentation is repeated and the mass dried on a Büchner funnel. The ion exchanger is washed with 2 liters fo 1 % sulfuric acid, followed by distilled water until the filtrate is neutral; and finally with 95 % ethanol, followed by ether. Before analysis the powder is dried over phosphorus pentoxide *in vacuo*. Sulfur determination and titration are performed as with sulfomethyl cellulose (see Fig. 1).

Triethylaminoethyl cellulose (*TEAE-cellulose*)

The influence of different factors involved in the ethylation has not been studied in detail. Preparation of the ionic exchanger was performed as follows:

Eighty grams of diethylaminoethyl cellulose prepared according to Peterson and

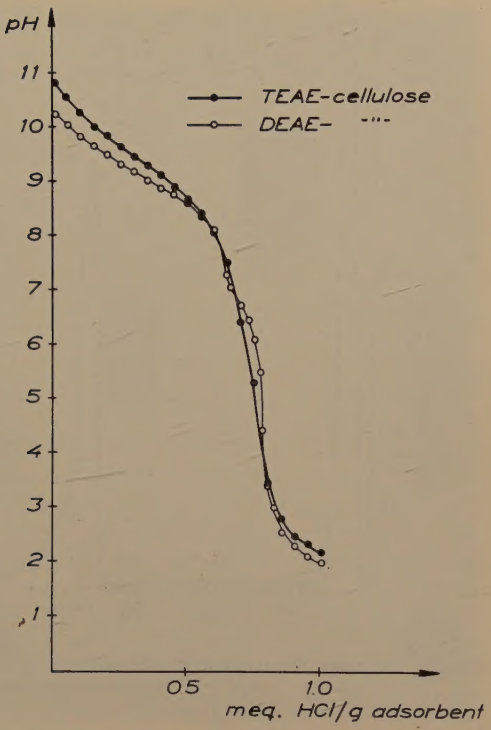

Fig. 2. Titration curves of DEAE- and TEAE-cellulose.

Sober were transferred to a 1 liter flask with a reflux condenser. 350 ml of 10 % ethylbromide in ethanol (*v/v*) was added, and the mixture boiled for 4 hours. The product which becomes somewhat more finely grained as a result of this treatment was dried and washed with 1 liter of water. The ion exchanger is preferably stored in wet or dry states in bromide form. If the free base is desired, the product is washed with one liter of 1 % aqueous NaOH solution, with distilled water to neutral reaction, and subsequently with acetone and ether. Prior to analysis the product was dried *in vacuo* over phosphorus pentoxide. Even slight heating of the free base *in vacuo* should be avoided, because of the risk of degradation.

Titration of the liberated di- and triethylaminoethyl cellulose products was performed according to Peterson and Sober (Fig. 2). After titration the acidic suspension was boiled and back-titrated. While carboxyl groups could be demonstrated in triethylaminoethyl cellulose even on back titration the titration curves of triethylaminoethyl cellulose showed in both cases that the concentration of free carboxyl groups must have been very low. Long exposure of the triethylaminoethyl cellulose to sodium hydroxide solution will naturally rupture all ester bonds which might have been formed by the ethylation process.

B. Chromatographic fractionation of equine anti-diphtheria serum

Equine anti-diphtheria serum (placed at my disposal by the State Bacteriological Laboratory, Stockholm) was dialyzed at 4° for 6 hours against sodium phosphate buffer (the ionic strength μ 0.005, pH 6.1). The resulting precipitate was removed

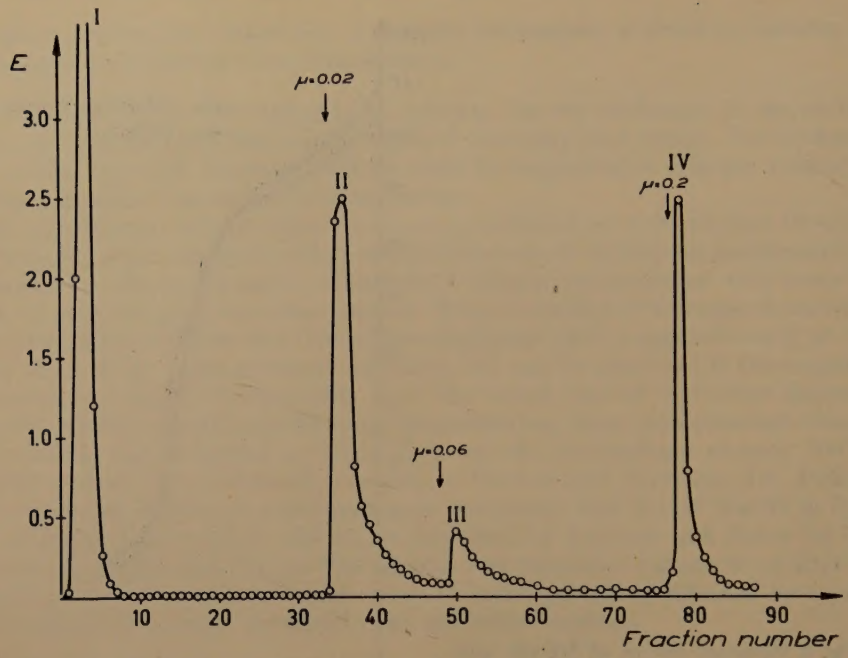

Fig. 3*a*. Stepwise displacement chromatogram of equine anti-diphtheria serum obtained with a sulfomethyl cellulose column.

by centrifugation. The clear supernatant solution was diluted with sodium phosphate buffer (μ 0.005, pH 6.1) so that the solution contained 1.75 mg N per ml. Six ml of this solution was placed on top of a column (8 × 0.9 cm) containing 2.1 g of sulfomethyl cellulose (dry weight) washed to equilibrium with sodium phosphate buffer (μ 0.005, pH 6.1). The chromatogram was developed with the same sodium phosphate solution at a rate of approximately 35 ml per hour. The eluate was collected in 3 ml fractions. When 100 ml solution had passed through the column, the concentration of the eluting agent was changed (μ 0.02, pH 6.1). After 45 ml of this buffer had passed into the column the concentration was again increased (μ 0.06, pH 6.1). Finally, after 75 fractions had been collected, displacement was effected by a buffer of even higher concentration (μ 0.2, pH 6.1).

The optical density of the fractions were measured in a Beckman DU spectrophotometer (1 cm light path) at 280 mμ with the weakest buffer solution as blank. The contents of the tubes corresponding to peaks numbered 1, II and IV (see Fig. 3*a*) were pervaporated to approximately $\frac{1}{2}$ ml. Ten μl of each fraction was then subjected to paper electrophoresis in a moist chamber apparatus (type LKB, LKB-Produkter, Stockholm) in veronal buffer (μ 0.05, pH 8.6) for 18 hours at 3.5 mA. After drying, the electropherogram was developed with bromphenol blue solution (Fig 3*b*).

Discussion

The cellulose ethers, the synthesis of which has been described above, may be regarded as long chain molecules consisting of β-glucopyranose units sparsely substituted with sulfoalkyl or quaternary ammonium groups. The cellulose chains are held

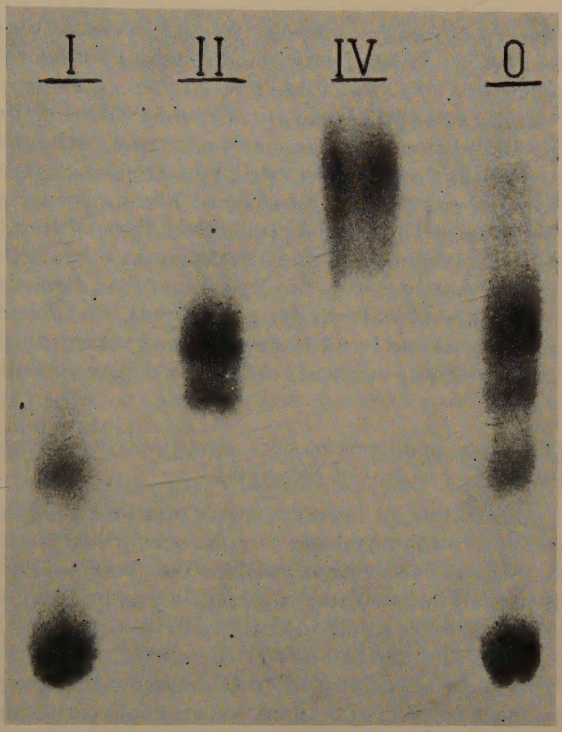

Fig. 3*b*. Paper electropherograms of the material in the peaks I, II and IV in Fig. 3*a* obtained in veronal buffer. O represents the original serum. Details are given in the text.

together by secondary forces—mainly hydrogen bonds—which reduce the solubility of the cellulose ethers. The irregular substitution of rather bulky side groups results, however, in a crystal or micelle structure which is less organized than that of cellulose itself. At the same time that a strongly hydrophilic group is introduced, thus reducing the interchain forces, the chains are moved apart and the substituents become freely exposed and thus become adsorptively active.

This makes possible a high adsorption capacity for proteins on slightly substituted cellulose derivatives of the ion exchange type. The sparse distribution can further be supposed to be of advantage and sometimes even necessary for protein chromatography.

Protolytic nature and ion exchange

Of utmost importance is the protolytic behaviour of the ion exchangers. Sulfoethyl cellulose has been titrated potentiometrically by Timell (6). Timell's titration curve is in good agreement in its general course with that reproduced in Fig. 1, although the lower branch lies approximately one pH unit lower than that found by him. The difference probably depends on the use by the present author of 0.5 molar sodium chloride solution in the titration while Timell seems to have titrated without addition of salt. Peterson and Sober (3) obtained widely different titration curves of other cellulose ion exchangers depending on whether titration was performed in the presence or

absence of sodium chloride. As may be seen from Fig. 1, the titration curve of the product synthesized with chloromethane sulfonate is very similar to that of sulfoethyl cellulose.

As a result of this strongly acidic character of the sulfonic ion exchangers in adsorption desorption processes, small changes in pH (a few tenths of a unit) at constant ionic strength as a rule cause only slight changes in the adsorption equilibrium. Displacement chromatograms of substances of low molecular weight as well as proteins appear therefore to be easily reproducible. Rate of development is not critical.

Diethylaminoethyl and triethylaminoethyl cellulose are rather strong bases. The former, in contrast to the latter, contains carboxyl groups if synthesis is carried out in the presence of oxygen (see Fig. 2). Certain differences in adsorption properties have been noted but it has not yet been determined whether these depend on different carboxyl group content, or on the degree of substitution of the amino groups.

Difference in adsorption characteristics between ion exchangers of cellulose type and those based on cross-linked polystyrene

Adsorption on ion exchangers of aromatic-alifatic net or chain structures depends not only on ion exchange but also on other factors. The close contact which occurs as a result of the formation of an ionic bond makes possible the development of strong dispersion forces between aromatic groups in the adsorptive and those in the adsorbent. This can result in irreversible adsorption. It may therefore be expected that cellulose derivatives are to be preferred when reversible adsorption of aromatic substances, especially those of high molecular weight, is desired. This supposition has been confirmed in many experiments carried out by the author and others. Some of them will later be reported in detail but a few examples will be mentioned. It is easy to demonstrate that, for example, dinitrophenylamino acids, which can not be satisfactorily chromatographed on Dowex 2, migrate as narrow zones on elution or displacement from diethylaminoethyl or triethylaminoethyl cellulose and can be recovered quantitatively from the latter in contrast to the former. The same observation has been made in folic acid fractionation experiments (11).

Another example may aslo be mentioned. Whereas a melanophore stimulating polypeptide (β-MSH), consisting of 18 amino acid residues, among which are tryptophyl and tyrosyl residues, has not as yet been successfully chromatographed on Dowex 50, it can easily be adsorbed and desorbed under mild conditions when sulfoethyl or sulfomethyl cellulose is employed. It should be stated though, that these differences may also depend on different charge densities.

Other factors

In addition to ion exchange and aromatic nuclei interaction, hydrogen bonds between the adsorbent and the adsorptives are likely to play a profound role in conditioning the chromatographic properties of the ion exchangers.

Chromatographic separations

A number of enzymes (12, 13, 14, 15), peptide hormones (16), and alder pollen allergen (17) have been purified using chromatography on sulfomethyl, sulfoethyl, and triethylaminoethyl cellulose. In all cases hitherto investigated, quantitative or

very high yields have been obtained, determined on the basis of activity as well as total amount of protein. It has also been shown that material purified by sulfonic acid ion exchangers can be further purified on amino ion exchangers and vice versa. A combination of these different types of adsorbents often seems to be more effective than the extended use of only one type of ion exchanger.

In the only chromatographic experiment described here, equine anti-diphtheria serum has been fractionated on sulfomethyl cellulose. The chromatogram (Fig. 3*a*) shows four distinct peaks corresponding to each increase in the buffer concentration. Paper electrophoresis of the material in the three main peaks (Fig. 3*b*) reveals that the first peak represents chiefly albumin and α-globulin, while the second represents β-globulins with diphtheria antitoxin (18), and the fourth peak represents γ-globulins. By re-chromatography of the material from Peak I on a column of suitable dimensions, albumin and α-globulin can easily be separated. By varying the load and choice of displacing buffers, and possibly by using gradient elution, a considerably sharper fractionation of serum proteins should certainly be obtained.

The example shows, however, that the adsorption in general follows inversely the order of the electrophoretic mobility at alkaline pH, while, as expected, the reverse conditions apply in diethylaminoethyl cellulose chromatography (4).

Sulfomethyl cellulose can be prepared very cheaply so that the use of this exchanger for the industrial fractionation of blood serum, for example, is certainly feasible.

Malmström (12) has fractionated enolase on sulfoethyl cellulose in Mg form. The strong affinity between enolase and magnesium seems to lend to the ion exchanger, which has been pretreated in this manner, the character of a more specifically directed adsorbent for enolase. Further experiments designed to study the applicability of this principle are in progress.

Comparison between cellulose ion exchange chromatography and preparative zone electrophoresis will be published later.

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A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*

I. The preparation of the factor inhibiting fertilization

By Ulla Esping

With 1 figure in the text

Harding (5) investigated the inhibiting capacity of heparin and some other acid polysaccharides upon fertilization of sea urchin eggs. Among other preparations, he used one from *Fucus vesiculosus* extracted by Dr. E. Vasseur. This preparation also contains the acid polysaccharide fuciodin. It was found to inhibit fertilization. Later it turned out, however, that more purified fucoidin preparations had no fertilization-inhibiting effect. The active substance might be a concomitant of fucoidin but it has a chemical composition different from that of fucoidin. Preparations similar to those used by Harding were afterwards tested on their inhibiting effect on fertilization of the sea urchin eggs by J. Runnström and his co-workers (9, 10).

The enzyme hyaluronidase plays probably a role in fertilization of mammals. Since fertilization inhibition may take place via enzyme inhibition it was of interest to investigate whether this enzyme is influenced by the *Fucus* preparations. Comparative experiments were first carried out by Drs. C. and D. Harding, Calif. Their results revealed that there was a good correlation between inhibition of fertilization of sea urchin eggs and the hyaluronidase-inhibiting effect. (See Table 1*a* and *b*.)

The hyaluronidase-inhibiting effect of different preparations has afterwards been used as a test in procedures of purification. Some of the most active preparations were also tested in experiments with sea urchin eggs.

The first part of this paper deals with the preparation of the fertilization-inhibiting factor from *Fucus vesiculosus*. Another type of fertilization inhibitor, which was also prepared from *Fucus vesiculosus*, has been investigated by Wicklund (10). This inhibitor was prepared by Dr. B. Lindberg from the same alga material, which was used for the preparations described in the present paper.

The latter part of this paper deals with analyses carried out on different fractions of extracts from *Fucus vesiculosus*.

I. Method for the preparation of the fertilization-inhibiting factor from *Fucus vesiculosus*

A method introduced by Black, Dewar and Woodwards (2) for preparation of fucoidin was used. This method was, however, modified by Dr. E. Vasseur. The inhibiting factor was then subjected to further purification. The mode of preparation varied somewhat in different extractions but has, on the whole, followed two lines.

Table 1a. Harding's preliminary results regarding the fertilization inhibition in sea urchin eggs.

Concentr. (%)	Per cent fertilization in various concentrations of						
	Sup C_1	Sup C_2	B_1	B_2	B_3	C_2	E_1
0.2	0	0	0	0	6	1	56
0.1	0	0	12	0	74	15	95
0.05	0	0	79	2		82	
0.025				14			

Table 1b. Preliminary results regarding the hyaluronidase-inhibiting effect of the splitting of hyaluronic acid.

Concentr. (%)	Per cent inhibition in different concentrations of							
	Sup C_1	Sup C_1 dial.	Sup C_2	B_2	B_3	C_2	C_3	E_2
0.03	90	80	80	80	5	10	5	10
0.003	5		10	5				

(*a*) The first five preparations were made as follows. The alga *Fucus vesiculosus* was collected at Kristinebergs Zoological Station on the Swedish West Coast, quickly rinsed in fresh water and airdried. The lot was immediately sent to Stockholm and was further dried at 20–25°C. The dried alga was ground in a ball mill. 200 g dried alga was extracted with 2000 cc distilled water. A reflux condensor was used. The extraction was done in an oil bath for approximately four and a half hours. Nitrogen was bubbling through the water–alga mixture to prevent oxidation. The liquid was decanted and centrifuged (4000 rpm). The sediment was added to the remaining alga and another extraction was made with the same volume of distilled water. The supernatant was kept under toluene in a cold place. The extraction was repeated four times. The five water extracts were mixed and evaporated to dryness, in vacuum in a water bath at approximately 40°C. The preparation was thereafter dried once more with phosphorus pentoxide in vacuum. Finally the remainder of the extracted alga was discarded. The dried preparation was extracted with 4 g sodium chloride in 800 cc water. Cooled (95.5 per cent) alcohol was added slowly, whilst stirring, until a final concentration of 30 per cent alcohol was reached. The suspension was left at +4°C over night and was afterwards cenrifuged. The precipitate was washed three times with 30 per cent alcohol (in water) which contained NaCl of equal concentration to that prevailing at the precipitation with alcohol. The wash liquid was added to the supernatant. The precipitate was dried a few times with 95.5 per cent alcohol and 2–3 times with ether. Finally the preparation (=B) was dried in vacuum over calcium chloride and blue gel.

The wash liquid and the supernatant obtained at the centrifugation were evaporated in vacuum at 40°C. Alcohol (95.5 per cent) was added to the solution until the concentration of alcohol was 60 per cent. This suspension was treated in the same way as that obtained with 30 per cent alcohol. The precipitate was called C.

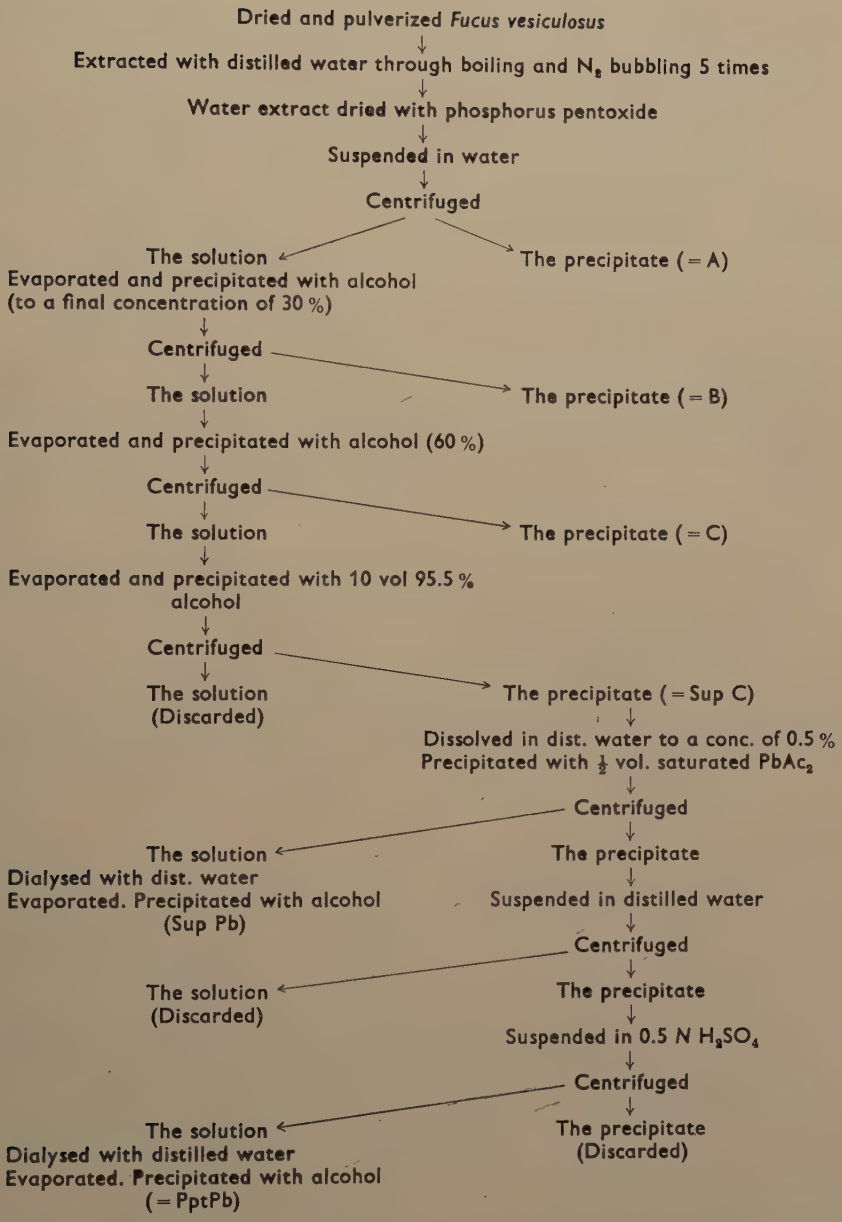

Fig. 1. Preparation of the *Fucus* substances.

The wash liquid and the supernatant were also evaporated after centrifugation and were then precipitated with 10 volumes of 95.5 per cent alcohol. This preparation (Sup C) turned out to be the most active one. However, it could be further purified by precipitation with a saturated solution of lead acetate in water.

The last preparation (Sup C) was dissolved in distilled water to a concentration of 0.5 per cent. Half the volume of saturated lead acetate was added to this solution slowly in the cold whilst stirring. A precipitate was formed. The suspension was centrifuged after having remained overnight in a cold room. The precipitation was washed with a solution of distilled water and saturated lead acetate (2:1). The supernatant was added to the wash liquids and was dialysed (passed through a cellophane membrane) with distilled water. The contents of the dialysis tube were evaporated in the customary way and were precipitated with 10 volumes of 95.5 per cent alcohol saturated with NaCl. The precipitate was called Sup Pb.

The precipitate with lead acetate was suspended in distilled water and was left to shake overnight in a cold room. The suspension was then centrifuged. The sediment was further treated with 0.5 *N* sulphuric acid. The precipitate which is undissoluble in sulphuric acid (mainly $PbSO_4$) was discarded. The supernatant and the wash liquid were dialysed with distilled water. The contents of the dialysis tube were evaporated to a small volume and were precipitated with ca. 10 volumes of 95.5 per cent alcohol saturated with NaCl in the usual way. This precipitate was called PptPb, and the preparation turned out to be the most active one obtained from *Fucus*.

(*b*) From the fourth preparation the alga was first extracted with ether and then with methanol. The remainder of the alga was extracted in water as described under *a*. This extract was further treated according to the same method.

Dr. B. Lindberg evaporated the methanol extract. After that the dry preparation was dissolved in water and precipitated with lead acetate. The lead precipitate, which contained phenols, was dissolved by bubbling sulphur dioxide through the suspension. The water solution thus formed passed through an ion exchange column. The phenols were separated from the carbohydrates by repeated precipitation with lead acetate (7).

(*c*) From the sixth preparation the procedure was changed somewhat. A precipitation was brought about at an alcohol concentration of 25 per cent (=B) and thereafter at those concentrations which gave obvious precipitates. This was at 52 per cent (=C), 57 per cent (=D) and 61 per cent (=E) alcohol. The solution after the last precipitate (=E) was evaporated and precipitated with lead acetate and treated as under *a*.

Tables 3 and 4 show that preparations obtained in this manner were always the most active. The subindices in the tables refer to the different preparations.

At each preparation the hyaluronidase-inhibiting capacity of various fractions was investigated. For methods see ref. 4.

II. Analyses

Methanol and ether extraction

Sup C_1 prepared as recorded previously (see above) was suspended in methanol to 0.33 per cent and extracted under reflux for five hours in a water bath. The suspension was filtered and the filtrate was evaporated to dryness. Sup C_1 was suspended in the same manner in ether (peroxide-free) to 0.25 per cent and extracted.

The supernatants and the evaporated filtrates were respectively dissolved to the

Table 2. The hyaluronidase inhibition of methanol- and ether-extracted preparations.

Preparation	Inhibition of hyaluronidase %
Methanol	
dissoluble part	15
undissoluble part	80
Ether	
dissoluble part	0
undissoluble part	100

same concentration in a solution of 0.9 per cent NaCl in water. These solutions were then tested as hyaluronidase inhibitors. It then turned out that the active part of the *Fucus* preparation was difficult to dissolve in methanol and that it was undissoluble in ether. See Table 2.

Dialysis

A solution of Sup C_1 was dialysed overnight with running water. In a cold room it was then dialysed with distilled water whilst stirring during another 12 hours. The distilled water was changed three times during the experiment. The solution in the dialysis tube was then diluted with a NaCl solution. The final concentration of Sup C_1 was 1 per cent and of NaCl 0.9 per cent. Dialysed as well as undialysed Sup C_1 of the same concentration (in 0.9 per cent NaCl) were tested as hyaluronidase inhibitors. Both solutions inhibited hyaluronidase about 80 per cent. The active component of the preparation must thus be regarded as undialysable.

Electrophoresis

Some very active substances were tested by means of electrophoresis. The preparations were dissolved to 2 per cent in phosphate buffer of pH 3.35 with the ion strength 0.1. When testing various cell contents subsequent to electrophoresis it turned out that the active component migrated towards the positive pole. Subsequent to the experiment the different cell contents were dialysed with distilled water. The content of every cell was diluted to the same extent. The concentration of NaCl in the solution was 0.9 per cent. These solutions were tested as inhibitors in the usual way. The original preparation (dissolved in 0.9 per cent NaCl) diluted to the same concentration as the cell contents, had an inhibitory effect of 45 per cent. The effect of the upper positive cell part was 20 per cent and of the lower positive part 35 per cent. The bottom had an effect of 35 per cent and the lower negative part 30 per cent. The upper negative part had no effect.

Three different migrating components could be discerned. One migrated towards the positive pole at a rate of 18.3×10^{-5} cm^2/volt sec., another migrated towards the positive pole at a rate of 11.4×10^{-5} cm^2/volt sec., and a third component migrated towards the negative pole at a rate of 8.5×10^{-5} cm^2/volt sec. These values all refer to Sup C_1.

Spectrophotometry

Spectrophotometric studies of the fractions were carried out on a Beckman spectrophotometer. No characteristic absorption peaks were found. The extinction curve was parallel to the abscissa between 600 mμ and 270 mμ. There the curve changed direction and became almost parallel to the ordinate. The extinction was measured between 600 and 215 mμ.

Determination of the colour strength

In some cases the colour strength seemed to be directly proportional to the enzyme-inhibiting activity of the preparation. The solutions thus varied in colour from pale yellow-brown to darkbrown. The colours of the solutions were compared. Since no colorimetric tools were used the determinations have to be regarded as rather subjective.

The colours of the solutions were estimated according to a scale of five steps. After the colour determination the hyaluronidase-inhibiting capacity of the preparations was tested. There was an obvious correlation between the colour intensity of the solutions and the inhibiting capacity. After treatment with sodium borohydride the colour dissapeared, however, without decrease in inhibiting capacity. A coloured contaminant, very difficult to remove, may perhaps be present. (Cf. 4.)

Paper chromatography

The active component could not be separated from contaminating substances by paper chromatography in butanol–water (6:1) or in butanol–glacial acetic acid–water (4:1:5). None of the components migrated.

Dr. E. Vasseur made some estimations of sugars on the hydrolysed *Fucus* preparations by aid of paper chromatography. He found that the dominating component was glucose. Fucose, xylose, and mannose were also found. Galactose was present only in very small amounts.

Fucose determination

The amount of fucose was determined in order to find out whether there was a correlation between this value and the enzyme-inhibiting effect. The fucose determinations were carried out according to the method of Dische and Shettle (3).

Sulphuric acid was added to the cooled test solution, slowly, whilst constantly stirring. It was then boiled during 10 minutes. After cooling, cysteine hydrochloride was added. A few hours later the extinction was measured at 399 and 430 mμ. The concentration of fucose could then be estimated by comparing with a standard curve. No correlation whatsoever was found between the hyaluronidase-inhibiting capacity and the fucose content. (See Table 3.)

According to the results of the electrophoresis experiments the enzyme-inhibiting component of the *Fucus* preparations must be an acid substance. Sulphur- or phosphorus-containing acid groups are probably present. In order to find out whether this is the case, estimations were made on the content of sulphur as well as of phosphorus.

Sulphate determination

The samples were hydrolysed with 0.5 *N* hydrochloric acid under reflux. They were subsequently neutralized with sodium hydroxide to pH 3–4.

Table 3. Comparison of the fucose content and the hyaluronidase inhibition.

Preparation	Fucose (%)	Inhibition of hyaluronidase	
		Conc. inhib. (%)	Inhibition (%)
B_1	12	1.44×10^{-2}	
C_1	30.8	1.44×10^{-2}	
Sup C_1	7	1.44×10^{-2}	80
B_2	11.6	1.44×10^{-2}	80
C_2	26.6	1.44×10^{-2}	100
Sup C_2	6.5	1.44×10^{-2}	80
B_3	8.8	1.44×10^{-2}	0
C_3	28.2	1.44×10^{-2}	5
Sup C_3	31.4	1.44×10^{-2}	40
Sup C_4I	31.0	2.87×10^{-3}	100
Sup C_4II	22.2	2.87×10^{-3}	95
Sup C_5I	13.7	2.87×10^{-3}	95
Sup C_5II	0.9	2.87×10^{-3}	95
$PptPb_5$	20.9	0.96×10^{-3}	90
B_6	9.6	2.87×10^{-3}	55
C_6I	33.8	2.87×10^{-3}	65
C_6II	33.7	2.87×10^{-3}	60
D_6I	33.6	2.87×10^{-3}	60
D_6II	36.1	2.87×10^{-3}	50
E_6I	26.0	2.87×10^{-3}	95
E_6II	25.2	2.87×10^{-3}	90
$PptPb_6$	7.4	2.87×10^{-3}	90
Sup Pb_6	27.6	2.87×10^{-3}	0
C_7IV	37.0	2.87×10^{-3}	20
D_7II	32.0	2.87×10^{-3}	45
E_7II	7.5	2.87×10^{-3}	75
$PptPb_7$	20.4	0.43×10^{-3}	50
$SupPb_7$	22.4	2.87×10^{-3}	0

The solutions were boiled and filtered. Barium chloride was added to approximately 3.5 per cent. The suspension was left in a cold room overnight. It was then filtered through a weighed and cooled porcelain filter crucible. The precipitate was washed with a small amount of distilled water at 0°C. It was thereafter dried with alcohol and then with ether. Finally the crucible with the precipitate was left in a thermostat for one hour at 110°C. The content of sulphur was somewhat varying in different preparations, but as a rule the preparations contained 3–6 per cent sulphate.

Phosphorus determination

Quantitative analyses on phosphorus have been done in some preparations. A spectrophotometric method was used. It was developed by Allen (1) and somewhat modified by Lindvall and Carsjö (8).

The samples were heated with perchloric acid in a salt bath at 160–180°C. They were subsequently diluted with water and ammonium molybdate and Amidol was

Table 4. Comparison of the nitrogen content and the hyaluronidase inhibition.

Preparation	Nitrogen (%)	Inhibition of hyaluronidase	
		Conc. inhib. (%)	Inhibition (%)
C_6I	0.5	2.87×10^{-3}	65
C_6II	0.6	2.87×10^{-3}	60
D_6I	0.5	2.87×10^{-3}	60
D_6II	0.4	2.87×10^{-3}	50
E_6I	0.5	2.87×10^{-3}	95
$PptPb_6$	1.6	2.87×10^{-3}	90
Sup Pb_6	0.3	2.87×10^{-3}	0
C_7IV	0.4	2.87×10^{-3}	20
D_7II	0.5	2.87×10^{-3}	45
E_7II	0.2	2.87×10^{-3}	75
$PptPb_7$	1.1	0.43×10^{-3}	50
Sup Pb_7	0.2	2.87×10^{-3}	0
B_8	0.6	2.87×10^{-3}	35
C_8II	0.6	2.87×10^{-3}	45
$PptPb_8$	1.1	2.87×10^{-3}	95
Sup Pb_8	0.3	2.87×10^{-3}	10

added. The extinction was finally measured in a Klett–Summerson photometer with a filter of 620 mμ. The phosphorus content was obtained from a standard curve. The different *Fucus* preparations contained approximately 0.06 per cent phosphorus.

Nitrogen determination

Quantitative analyses of nitrogen were carried out on each fraction obtained from some of the extractions. These nitrogen values have been compared with the corresponding values of the inhibition of hyaluronidase.

The method used for quantitative determination of nitrogen has been described by Lindvall and Carsjö (8). The tests were combusted with sulphuric acid with added H_2O_2. The reaction was carried out in a salt bath at 180–200°C. Upon oxidation the tests were diluted and Nessler reagent (6) was added. The extinction at 430 mμ was read in a Beckman photometer. The nitrogen concentration could finally be obtained from a standard curve.

The Sup Pb preparations which were less active as enzyme inhibitors contained the smallest amount of nitrogen, around 0.2–0.3 per cent (Cf. Table 4). The fractions which contained the greatest amount of nitrogen were PptPb. These held throughout a nitrogen concentration above 1 per cent. $PptPb_6$, for instance, contained 1.6 per cent of nitrogen. These preparations were more active than most others. However, it is not certain that there is a correlation between the nitrogen content and the hyaluronidase-inhibiting capacity. Certain preparations like E_6I with a low nitrogen content nevertheless strongly inhibited hyaluronidase. It is therefore quite likely that *Fucus* preparations contain a nitrogen-rich contaminating substance, which only with difficulty is separated from the active component (see Table 4).

SUMMARY

It has so far been impossible to obtain a pure preparation of the enzyme-inhibiting substance occurring in extracts from *Fucus vesciculosus*. Only some few characteristics of the active component are known, about the qualities of which the following may be mentioned.

It seems to be difficult to dissolve the active component of the *Fucus* factor in organic solvents as methanol and ether.

The molecular weight is so great that the substance cannot be dialysed (passed) through a cellophane tube.

The active component cannot be separated from the defilements by aid of paper chromatography.

Upon hydrolysis glucose, fucose, xylose, mannose, and galactose were found in the *Fucus* preparations.

The hyaluronidase-inhibiting component is an acid substance. In electrophoresis experiments it migrates to the positive pole at pH 3.35.

The *Fucus* preparations have no characteristic extinction curve. The solution tested had, however, a colour intensity which as a rule was parallel to the inhibiting effect.

The substance contains 3–6 per cent sulphate, and the acid groups may therefore be sulphate groups.

The preparations also contain phosphorus (around 0.05 per cent).

The hyaluronidase-inhibiting activity does not seem to show any connection with the fucose content.

It is also uncertain whether there is a correlation between the hyaluronidase inhibition and the nitrogen content.

The active principal is most likely of a polyphenol character.

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I want to express my gratitude to Dr. Erik Vasseur for directing me during the preparation and the analyses of the fertilization inhibitor. I am much indebted to Professor John Runnström for this encouraging interest in these investigations. At the same time I regret that this work had to be interrupted for reasons outside my control, but it is my hope that the data presented will serve as a basis for further experiments in this direction.

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A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*

II. The effect of the factor inhibiting fertilization on some enzymes

By Ulla Esping

With 1 figure in the text

It has been demonstrated that heparin and other similar polysaccharides inhibit cell division (8, 9). Roth (27) suggested that these substances may act by interfering with ribonuclease (RNase). Harding studied the action of heparin and of extracts from the alga *Fucus vesiculosus* (called fucoidin) on fertilization in the sea urchin egg (28). Wicklund further investigated the fertilization-inhibiting effect of similar extracts from *Fucus* [called FeInh(Fu)] (32).

Spermatozoa from Mammalia contain hyaluronidase. This enzyme facilitates fertilization (28). Lundblad and Monroy have also described a weak hyaluronidase activity in sea urchin sperm (18). Krauss (13) was, however, not able to find any hyaluronidase activity in sea urchin sperm.

The purpose of this paper is to present data concerning the inhibition of RNase and hyaluronidase by substances obtained from *Fucus vesiculosus*. This activity is compared with that of various other inhibitors of known composition. Data are also presented which show that the inbibiting substance from *Fucus* is not enzyme specific.

Material and methods

Inhibitors

The inhibitor from *Fucus vesiculosus* was prepared from the dried alga. The algae were ground and extracted in nitrogen atmosphere with distilled water kept boiling by an oil bath of about 130°C. The suspension was fractionated with alcohol. The fraction obtained when more than 60 per cent alcohol was used proved to be the most active. The method was described in the preceding paper (7).

The chondroitin sulfuric acid was obtained from Dr. Gardell. It was dissolved in water and reprecipitated with alcohol. The pectic substance and alginic acid were obtained from Dr. Säverborn and Dr. Öy, respectively, and were purified by Immers (11). The chitin disulfuric acid and cellulose trisulfuric acid were prepared by Astrup (2, 3). Other inhibitors used were: NaCu chlorophylline (Rystan Co.) (4), polyphloretinphosphate (LEO) (6), heparin (Vitrum), resorcinol (Baker's Analyzed), dihydroxyquinone (Baker), pyrogallol (Merck), gallic acid (D.A.B. 6), tannic acid (Schering-Kahlbaum), germanin (Bayer), quinone (Merck), pyrocatechol (Merck), phloroglucinol (Eastman Kodak), and naphthoresorcinol (Hoffman La Roche).

Determination of ribonuclease activity

Ribonucleic acid becomes more soluble as the time of RNase incubation is increased. The amount of acid soluble phosphate was taken as a measure of the enzyme activity. The activity was determined according to Kunitz (14).

Yest nucleic acid from Baker was purified by reprecipitation with glacial acetic acid (14). It was suspended in a 0.1 *M* acetate buffer of pH 5.0. Fresh suspensions were made every day. RNase (Worthington Biochemical Sales Co.) was dissolved in 0.01 *M* acetate buffer of pH 4.0. The enzyme and inhibitor were mixed for five minutes. In the controls enzyme and solvent were mixed. The time for enzyme incubation was three hours at 37°C. The concentration of RNase in the reaction mixture was 2.5×10^{-4} and of ribonucleic acid 0.1 per cent.

The addition of a solution of 0.25 per cent uranium acetate in 2.5 per cent trichloracetic acid stopped the enzyme reaction. The precipitate was removed by passing through a filter 30 minutes after the addition of the acid. The total amount of phosphate in the solution was determined by the procedure of Allen (1). This method was modified according to Lindvall and Carsjö (17). Non-incubated ribonucleic acid, RNase and inhibitor contained only negligible amounts of acid soluble phosphate. The per cent inhibition was calculated according to formula (I)

$$\text{Per cent inhibition} = 100\left(1 - \frac{x}{y}\right), \qquad \text{(I)}$$

x = the amount of acid-soluble phosphate in the substrate–enzyme–inhibitor mixture after three hours of enzyme incubation,

y = the amount of acid-soluble phosphate in the substrate–enzyme mixture after three hours of enzyme incubation.

Determination of hyaluronidase activity

The hyaluronidase activity was determined in Ostwald viscosimeters at 25°C.

The hyaluronic acid was dissolved in McIlvaine buffer of pH 7.0 (consisting of 12.75 g Na_2HPO_4, 2.02 g citric acid, and 3.5 g NaCl in 1000 ml distilled water). The concentration of the hyaluronic acid was adjusted to the desired viscosity. The time for the surface of the hyaluronic acid solution to run between the two marks of the viscosimeter was twice that of water. The enzyme was dissolved in a 0.9 per cent sodium chloride solution. Its concentration in the reaction mixture was 0.36 V.R.U./ml (Viscosity reducing units = V.R.U.[1]). The enzyme and the substrate were prepared by LEO. Malmgren has studied the properties of testis hyaluronidase from LEO (21). The inhibitor was incubated ten minutes with the enzyme. As a control the same amount of the enzyme and the solvent of the inhibitor were mixed. 0.1 ml of these solutions were then added to the substrate in the viscosimeter. The time for the surface of the solution to run between the two marks (the flow time) was measured many times during the first hour. The Rodney formula (II) was used in calculating the inhibition (26).

$$\text{Per cent inhibition} = 100\left(1 - \frac{y-x}{y-z}\right). \qquad \text{(II)}$$

[1] One V.R.U. is defined as the amount of enzyme, which will reduce the viscosity of the substrate used by one half in 10 minutes.

The difference in flow time between 0 and 30 minutes for the substrate–enzyme–inhibitor mixture $(y - x)$ was divided by the difference for the substrate–enzyme mixture $(y - z)$. All values were divided by the corresponding water values of the viscosimeters.

Determination of urease activity

Preparations from *Fucus* were tested as inhibitors of urease. The activity of urease was determined according to Sumner and Sisler (31). The enzyme (Pharmacia) was made up in a 2 per cent solution of gum arabic at pH 6.2. The icubations were performed at pH 6.0 (citrate buffer) at 45°C. The breakdown of the urea by the enzyme was stopped and the quantity of ammonia formed was determined by nesslerization. Neither urea and inhibitor nor urease and inhibitor were found to contain any free ammonia nitrogen. The per cent inhibition was calculated from formula (I).

x = The amount of free nitrogen in the substrate–enzyme–inhibitor mixture after three hours of enzyme incubation,
y = the amount of free nitrogen in the substrate–enzyme mixture after three hours of enzyme incubation.

The concentration of urea (Baker's Analysed) was 0.0903 and that of enzyme 3.6×10^{-3} per cent.

Determination of α-amylase acitivity

The activity of α-amylase was determined in the same way as that of hyaluronidase. The starch solution was prepared from Analar Starch soluble (BDH) by the method described by Hultin (10). The enzyme was prepared by Hultin. The determinations were made at 25°C, pH 5.2. Ostwald viscosimeters were used. The inbition was calculated by the Rodney formula (2). The x- and z-values represent the flow time 20 minutes after enzyme incubation. The concentration of the enzyme was 1.4×10^{-3} per cent and of the substrate 2.9 per cent.

Experiments and results

In order to determine the enzyme-inhibiting effect of *Fucus* preparations as compared with that of other inhibitors several different substances were tested. Some experiments were also made to determine the chemical nature of the preparations. As the molecular weights of most of the substances used were unknown, the per cent of the inhibitors in the solution was calculated for each substance. The active component of these *Fucus* preparations cannot dialyse (pass through a cellophane membrane) under ordinary conditions. With the flavonoids tested (rutin and hesperidin) it was impossible to make the solutions strong enough to obtain 100 per cent inhibition. As the preparations from *Fucus* are rich in polysaccharides and as heparin is said to be an inhibitor of RNase and hyaluronidase (20, 27) several acid polysaccharides were tested. It was also of interest to compare the effect of the inhibitors with the effect of polysaccharides of known strength (2).

Kylin (15) found a substance in *Phaeophyceae* related to tannins. This substance is not a tannin in the usual sense, but it has the same properties when tested against proteins. Tannins and phenols with three adjacent hydroxyl groups inhibit the en-

Table 1. Inhibition of hyaluronidase.

Inhibitor	Concentration of inhibitor in per cent $\times 10^3$	Inhibition (%)
Chitin disulfuric acid	1.43	10
Cellulose trisulfuric acid	14.3	20
Sodium alginate	14.3	15
Chondroitin sulfuric acid	14.3	25
	2.87	10
	0.72	0
Heparin	14.3	10
Resorcinol	143	80
	14.3	0
Dihydroxyquinone	2.87	0
Pyrogallol	2.87	0
Gallic acid	2.87	0
Tannic acid	2.87	100
	0.56	95
	0.11	40
	0.02	5
Germanin	2.87	50
Polyphloretinphosphate	0.143	50
	0.072	30
Chlorophylline	2.87	55
	1.43	30
Fucus preparation (Sup C_4I)	2.87	75
	0.56	30
	0.11	5
	0.02	0

zymes. The values found for the inhibition of hyaluronidase are given in Table 1 and for the inhibition of RNase in Table 2.

Several polysaccharides were investigated with respect to their inhibition of enzymes, but only heparin proved to be as effective as the inhibitors from *Fucus*.

All fractions from one extraction from *Fucus* were tested as enzyme inhibitors. The effect on α-amylase, hyaluronidase, and urease was determined. The inhibition (in per cent) was calculated (see under Materials and methods). The results are summarized in Table 3.

The inhibiting action of FeInh(Fu) upon fertilization and membrane formation in the sea urchin is removed by addition of sodium periodate (29, 32). In the same way oxidation of heparin with small amounts of sodium periodate removes its inhibiting action upon fertilization (8).

Fucus preparations, tannic acid and heparin were treated with sodium periodate (sodium meta periodate, G. F. Smith and Co.) at pH 7.0 for 5 hours at room temperature. The concentration of the inhibitor was 0.1 per cent and of sodium periodate 0.08 *M*. Controls were run in exactly the same way but the sodium periodate was exchanged by distilled water. Heparin was not destroyed by periodate of this concentration. The inhibiting effect of the *Fucus* preparations was diminished and that of tannic acid was destroyed. The *Fucus* preparations were not influenced by 0.08 *M* sodium periodate at pH 3.6 whereas tannic acid was (24). After the dialysation

Table 2. Inhibition of RNase.

Inhibitor	Concentration of inhibitor (%)	Inhibition (%)
Chondroitin sulfuric acid	2.5×10^{-2}	0
Sodium alginate	2.5×10^{-2}	0
Hyaluronic acid	2.5×10^{-2}	0
Heparin	2.5×10^{-2}	75
	2.5×10^{-3}	60
	2.5×10^{-4}	25
	2.5×10^{-5}	0
Pectic substance	2.5×10^{-2}	0
Quinone	2.5×10^{-2}	5
Dihydroyxquinone	2.5×10^{-2}	0
Pyrocatechol	2.5×10^{-2}	0
Resorcinol	2.5×10^{-2}	0
Pyrogallol	2.5×10^{-2}	50
	2.5×10^{-3}	40
	2.5×10^{-4}	0
Gallic acid	2.5×10^{-2}	30
Phloroglucinol	2.5×10^{-2}	0
Tannic acid	2.5×10^{-2}	100
	2.5×10^{-3}	70
	2.5×10^{-4}	20
	2.5×10^{-5}	5
	2.5×10^{-6}	0
Fucus preparation (Sup C_4I)	2.5×10^{-2}	95
	2.5×10^{-3}	60
	2.5×10^{-4}	15
	2.5×10^{-5}	0

To compare the effect per mol some substances of known composition were examined. The concentration of the inhibitor was 0.0025 *M*.

Gallic acid	50 %
Pyrogallol	55 %
Naphthoresorcinol	5 %

Table 3. Inhibition of α-amylase, hyaluronidase and urease.

Inhibitor	Inhibition in per cent		
	α-amylase	Hyaluronidase	Urease
B_6	0	15	0
C_6I	0	10	0
D_6I	0	0	0
E_6I	10	30	0
$PptPb_6$	20	80	35
Sup Pb_6	0	0	0

The per cent concentration of the inhibitor was in the α-amylase experiments 2.87×10^{-3}, in the hyaluronidase experiments 1.43×10^{-3}, and in the urease experiments 7.2×10^{-3}.

Table 4. Inhibition of hyaluronidase by preparations treated with periodate at pH 7.

Inhibitor	Concentration of inhibitor (%)	Inhibition in per cent	
		Untreated	Treated with periodate
Tannic acid	1.00×10^{-3}	70	5
Fucus prep. (Sup C_4I)	2.87×10^{-3}	40	30
	1.00×10^{-3}	20	5

Table 5. Inhibition of RNase by preparations treated with periodate at pH 7.

Inhibitor	Concentration of inhibitor (%)	Inhibition in per cent	
		Untreated	Treated with periodate
Tannic acid	1.25×10^{-2}	80	10
Fucus prep. (Sup C_4I)	1.25×10^{-2}	90	80
Heparin	1.25×10^{-2}	65	65

against distilled water the inhibitor solutions were adjusted to a desired concentration. In the RNase experiments they were diluted with distilled water and in the hyaluronidase experiments with a solution of sodium chloride in distilled water. The inhibiting values are shown for hyaluronidase in Table 4 and for RNase in Table 5.

Sodium borohydride has been used for the reduction of aldoses and ketoses (16). Sodium borohydride (Metalurg Inc.) dissolved in sodium hydroxide was mixed with boric acid and inhibitor. In the control only its solvent, sodium hydroxide, was used instead of sodium borohydride. The colour of the *Fucus* peparation changed from brown to yellow when added to the reducing agent. The solutions were allowed to stand for two hours at room temperature and then mixed with 2 *N* sulfuric acid (when a vigorous evolution of hydrogen took place) and then dialysed against distilled water saturated with nitrogen. In the reaction mixture the concentration of the inhibitor was 0.30 and 0.15 per cent and the concentration of the sodium borohydride 0.21 per cent. After the solutions had been adjusted to proper strengths, they were tested as enzyme inhibitors. Heparin, tannic acid, and *Fucus* preparations were not influenced by sodium borohydride. The results are given in Tables 6 and 7.

Some experiments were made in which the *Fucus* preparations were first treated with sodium periodate and then with sodium borohydride. The preparations from *Fucus* were treated with sodium periodate. After dialysis the hyaluronidase inhibition of experiment and control was determined. The effect was the same for both periodate-treated sample and control. The two solutions were then reduced by sodium borohydride in the usual way. The inhibiting activity of the periodate-treated solution was diminished (Table 8).

Serum albumin is able to counteract the inhibiting effect of the preparations from *Fucus* (30, 32) and of heparin. The inhibitor solution was mixed with the same volume of protein or amino acid solution. Some other substances were also tested. In the

Table 6. Inhibition of hyaluronidase by substances treated with borohydride.

Inhibitor	Concentration of inhibitor (%)	Inhibition in per cent	
		Untreated	Treated with borohydride
Tannic acid	1.43×10^{-3}	60	70
	0.29×10^{-3}	20	35
Fucus prep. (Sup C_4I)	1.43×10^{-3}	35	35
	0.29×10^{-3}	15	15

Table 7. Inhibition of RNase by substances treated with borohydride.

Inhibitor	Concentration of inhibitor (%)	Inhibition in per cent	
		Untreated	Treated with borohydride
Tannic acid	2.5×10^{-2}	50	50
Fucus prep. (Sup C_4I)	2.5×10^{-2}	90	90
Heparin	2.5×10^{-2}	80	80

Table 8. Inhibition of hyaluronidase by *Fucus* preparations (Sup C_4I) first treated with periodate and then with borohydride.

Treatment	Inhibition in per cent
Periodate and borohydride	10
Periodate	25
Borohydride	25
Control	25

controls the inhibitor was mixed with distilled water. After five minutes these mixtures were added to the enzyme.

The proteins, amino acids, and the other substances used were: clupeine sulfate (BDH), serum albumin (crystalline albumin from Armour Laboratory), gelatine (Fisher Scientific Co.), silk peptone (Hoffman La Roche), *p*-amido acetophenon (Hey C p.a.), guanidincarbonate (Merck), lecithin (animal 90 % Pfanstiehl), and *l*-histidine.

The results of the hyaluronidase experiments are summarized in Table 9. The amino acids or proteins alone had no effect on the hyaluronidase activity except betainhydrochloride which inhibited hyaluronidase. Serum albumin in the concentrations used activated the RNase. The results are shown in Table 10.

Some experiments were made in which the hyaluronidase was added to the hyaluronic acid, which was mixed with the *Fucus* preparation. In the controls the experiments were made in the usual way (the enzyme and the inhibitor were mixed before the mixture was added to the substrate). In these experiments the inhibition was not so high when the inhibitor had not been incubated with the enzyme.

Table 9. Inhibition of hyaluronidase by inhibitor (concentration 1.43×10^{-3} per cent) and protein or some other substances.

Inhibitor	Protein or other substances		Inhibition in per cent	
	Substance	Concentration (%)	With other substances	Without other substances
Fucus prep.				
($PptPb_6$)	Clupeine sulfate	7.2×10^{-3}	5	80
	Serum albumin	35.8×10^{-3}	0	80
($PptPb_8$)	Gelatine	28.6×10^{-3}	0	55
	Silk peptone	14.3×10^{-3}	75	55
	p-amido acetophenon	saturated	55	55
	Lecithin	saturated	50	55
	Guanidin carbonate	14.3×10^{-3}	50	55
	Betainhydrochloride	14.3×10^{-3}	90	55
	Histidine	14.3×10^{-3}	55	55
Tannic acid	Serum albumin	28.6×10^{-3}	10	95
Chlorophylline	Serum albumin	14.3×10^{-3}	0	30

Table 10. Inhibition of RNase by inhibitor and serum albumin.

Inhibitor Concentration 1.25×10^{-2} (%)	Serum albumin Concentration (%)	Inhibition in per cent	
		With protein	Without protein
Tannic acid	1.25×10^{-2}	95	95
	1.25×10^{-1}	0	95
Fucus prep. (Sup C_4I)	1.25×10^{-2}	50	80
	1.25×10^{-1}	0	80
Heparin	1.25×10^{-2}	60	80
	1.25×10^{-1}	Activation	80
Chlorophylline	1.25×10^{-1}	Activation	20
Pyrogallol	1.25×10^{-1}	0	45

Some hyaluronidase experiments were made in which the ionic strength was kept constant but in which the pH was varied. Three different kinds of buffers were used, acetate, citrate and phosphate. The pH was varied between 3.2 and 7.7, a range in which the enzyme was active. The inhibition values varied depending upon the buffers which were used. In every buffer the inhibiting effect was much higher at a lower pH. From pH 5 to 3 it changed from 40 to 80 per cent. Between pH 8 and 5 the inhibition values were almost constant.

The inhibition of hyaluronidase was measured by the usual method. This method is simple to use but gives only relative values. In this way it was possible to compare the inhibiting effect of the different substances. It is thus good enough to test the activity of substances before and after purification. But this method gives no absolute values. Futhermore it is not possible to calculate the effect of the enzyme at different pH. Thus Fig. 1 only shows the tendency. The highest enzyme activity was called 100. All the other values were compared with this value.

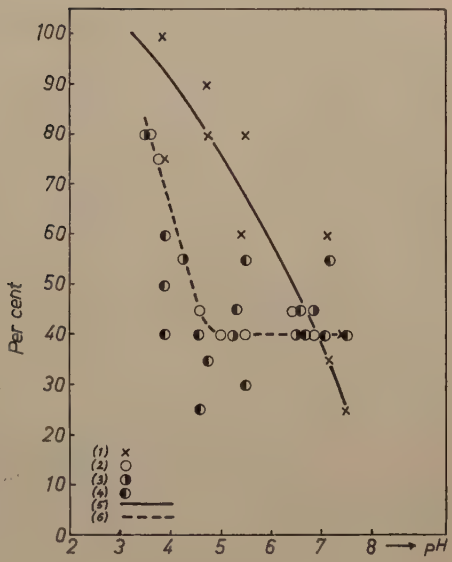

Fig. 1. Inhibition of hyaluronidase by *Fucus* preparation ($PptPb_9$) in different buffers at different pH. Ionic strength 0.1.

(1) Activity of the enzyme at different pH;
(2) inhibition (in per cent) in acetate buffer;
(3) inhibition (in per cent) in citrate buffer;
(4) inhibition (in per cent) in phosphate buffer;
(5) activity of the enzyme at different pH;
(6) inhibition of the enzyme at different pH.

Discussion

In the presence of sodium chloride, heparin loses its inhibiting effect on hyaluronidase (23). As McIlvaine buffer contains sodium chloride, it was impossible to get inhibition of hyaluronidase with heparin when this buffer was used. The enzyme inhibiting substance from *Fucus* was able to inhibit hyaluronidase even in the presence of sodium chloride.

The preparations from *Fucus* are very impure. It is therefore possible that one component inhibits urease and another hyaluronidase. In the experiment in which all fractions from one *Fucus* extraction were tried as inhibitors of α-amylase, urease, and hyaluronidase, the fraction which had the greatest inactivating effect on hyaluronidase also had the strongest inhibiting effect on α-amylase and urease. This, as well as the fact that periodate and borohydride treatment had the same effect on the inhibition of RNase as well as of hyaluronidase, substantiates the impression that the same component inhibited all the enzymes.

When the hyaluronidase was added to the mixture of hyaluronic acid and *Fucus* preparation, the inhibition was not so strong as when the enzyme and inhibitor were mixed before the incubation of the substrate. This indicates that the *Fucus* inhibitor acts on the enzyme and not on the substrate.

Some polysaccharides are destroyed even by low concentrations of sodium periodate. The experiments showed that the *Fucus* inhibitor was only broken down by sodium periodate of high concentrations. This does not indicate whether the active component is a polysaccharide or not. Hyaluronic acid (22), heparin (5) and some other polysaccharides are not easily broken down by periodate. On the other hand, several other substances, e.g. tannic acid, are destroyed by such treatment. With sodium borohydride the aldoses and ketoses are reduced. The experiments showed that such groups were not necessary for inhibition.

Preparations first treated with sodium periodate in such concentrations that their

inhibiting power was not diminished and then with sodium borohydride inhibited less than preparations treated with sodium periodate and then sodium hydroxide (control for borohydride treatment). This suggests that periodate changes the inhibitor so that borohydride has an opportunity to attack.

Runnström and Wicklund have shown (30, 32) that albumin can counteract the inhibition of fertilization caused by FeInh(Fu) and heparin if the solutions are mixed. The present investigation showed that not only serum albumin but also several other proteins have the ability to counteract the inhibition of enzymes. However, silk peptone and all other substances tested, *p*-amido acetophenon, lecithin, guanidin carbonate, betainhydrochloride, histidine had no effect upon enzyme inhibition.

Runnström and Wicklund are of the opinion that heparin and jelly substance are probably inhibitors of several different enzyme systems (30). Lundblad has investigated the proteolytic enzymes of the sea urchin egg (19). He found three enzymes with pH optimum not far from 7 called EI, EII and EIII. Of these enzymes EII and EIII were inactivated by preparations from *Fucus*. EI was not influenced. Thus the inhibitor from *Fucus* was not enzyme specific. Its inhibiting action was lost by the addition of proteins. These two facts indicate that the inhibitor is working on a non specific protein part of the enzymes. The simplest substance found in this investigation which inhibited in this way was pyrogallol. Rodney *et al.* are of the opinion that the flavonoids are unspecific inhibitors.

Heavy, negatively charged ions, e.g. tannic acid, precipitate proteins. It is necessary to use higher concentrations (in per cent) of the *Fucus* preparations than of tannic acid to obtain an inhibition. It is thus possible that the substance from Fucus in the concentrations used cause a denaturation of the proteins in a similar way as does tannic acid. This may perhaps explain the fact that both substances act on every enzyme tried. The effect of proteins may also support this interpretation.

Testicular hyaluronidase has its pH optimum below 5.4 (25). Electrophoretic studies showed that the active component of the *Fucus* preparations is negatively charged. It is most active in solutions with a pH lower than that which is optimum for the enzyme. This indicates that the *Fucus* inhibitor reacts with the alkaline groups, e.g. NH_2 group, of the protein. In a similar way trichloracetic acid, tannic acid and phosphotungstic acid are most active for precipitation of proteins in solutions of a pH lower than the isoelectric point of the protein. Heavy metals are most active at a higher pH. They react with the acidic groups particularly.

Wicklund, Kriszat and Runnström (33) showed that sea urchin eggs treated with *Fucus* preparations do not elongate in the centrifugal field to the same extent as do untreated eggs. If the *Fucus* preparations denaturate the proteins in the egg surface, this might explain the increased rigidity of the egg and the reduced elongation in the centrifugal field.

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I am very much indebted to Dr. Erik Vasseur for his valuable support, for discussions and criticism. I express my gratitude to Professor John Runnström for never-failing interest in my work. I wish to thank Dr. B. Högberg, LEO, for providing the pure hyaluronic acid and the hyaluronidase.

The Wenner-Gren Institute for Experimental Biology, Stockholm.

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Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 14

Communicated 27 February 1957 by Hugo Theorell and Erik Jorpes

Determination of amino acids as phenyl thiohydantoin derivatives[1]

I. Micro synthesis of 3-phenyl-2-thiohydantoins from amino acids

By John Sjöquist

With 5 figures in the text

The present paper describes a procedure for the quantitative synthesis of the 3-phenyl-2-thiohydantoins (PTH) derivatives of amino acids occurring in protein hydrolysates. *A priori* PTH derivatives should offer favorable possibilities in amino acid analysis because of (1) easy quantitative determination, owing to the high extinction in U. V., (2) good solubility in organic solvents, permitting easy separation from inorganic salts, (3) direct applicability to quantitative N-terminal amino acid analysis.

The synthesis involves the following reactions:

Stage I: $C_6H_5 \cdot NCS + H_2N \cdot CHR \cdot COOH \xrightarrow{pH\ 8\text{–}10} C_6H_5 \cdot NH \cdot CS \cdot HN \cdot CHR \cdot COOH$

I

Stage II: $C_6H_5 \cdot NH \cdot CS \cdot HN \cdot CHR \cdot COOH \xrightarrow{H^+} C_6H_5 \cdot N(OC)(NH)(CS)CH(R) + H_2O$

II

I = Phenylthiocarbamyl (PTC) amino acid.
II = Phenyl thiohydantion (PTH).

Materials

Acetone. The technical quality was refluxed for 3 hours with potassium permanganate, and afterwards stored in contact with the same agent. The desired quantity was distilled off immediately before use. B.p. 56°C.

[1] This paper forms part of a series of reports from this laboratory on the use of phenyl thiohydantoins in qualitative and quantitative amino acid analysis (1, 2, 3).

Phenyl isothiocyanate. Eastman Kodak. Distilled *in vacuo.*

Triethylamine. Eastman Kodak. This was refluxed for three hours with phtalic acid anhydride (5 g/100 ml). The desired quantity was distilled off on a Widmer column immediately before use. B. p. 89°C.

Acetic acid. Merck, Analytical Reagent. Distilled at ordinary pressure. B. p. 118°C.

Hydrogen chloride. Generated by pouring sulfuric acid into concentrated hydrochloric acid, and washed by passing the gas through concentrated sulfuric acid.

The acetic acid was thoroughly saturated in a quartz tube with hydrogen chloride at room temperature.

Ethanol, 95 p.c., having a negligible absorption down to 225 mμ, was used as the solvent in the spectrophotometry.

Amino acids were the commercial products of Merck & Co. except L-hydroxyproline (Hoffman la Roche) and L-cysteic acid (Light & Co.). All specimens gave satisfactory analyses for carbon, hydrogen and nitrogen.[1]

Glass-distilled water was used throughout.

Buffer. This was made up from 2.00 ml 2 *N* acetic acid, 1.20 ml triethylamine and distilled water to 25 ml. When mixed with an equal volume of acetone a pH of 10.1 resulted.

All synthetic operations were carried out in tubes made of transparent quartz, length 100 mm and inner diam. 8–9 mm. The tubes were cleaned in ethanol and ignited.

Methods

All measurements of light absorption were made in a Beckman Spectrophotometer, Model DU, with the use of matched quartz absorption cells ($d = 1$ cm).

Paper chromatography was carried out as described by Edman and Sjöquist (3).

Experiments and results

A. *Measurement of U.V.-absorption characteristics of PTH's*

The U.V.-absorption curve of PTH-valine is presented in Fig. 1. The absorption of the PTH's is generally characterized by a maximum around 269 mμ and a minimum around 245 mμ, although the relative heights of the maxima and minima vary for the individual PTH's. The absorption of the various PTH's has been measured at these two key wave lengths and the results are summarized in Table 1.[2]

B. *Synthesis of PTC derivatives (Stage I)*

For these synthesis 0.5 μmol of each amino acid (suitable limits: 10–100 μg) was generally used. In order to convert the amino acids to hydrochlorides, thereby simulating an acid protein hydrolysate, they were dissolved in 0.1 N hydrochloric acid and an aliquot containing the desired quantity was transferred to a quartz tube and excess of hydrochloric acid subsequently removed by

[1] The analyses were made at the Department of Analytical Chemistry, University of Lund, Lund, Sweden.

[2] The specimens used in this measurement were those synthesized by Edman (4, 5) except PTH-serine and PTH-threonine, which were synthesized essentially as described by Ingram (6).

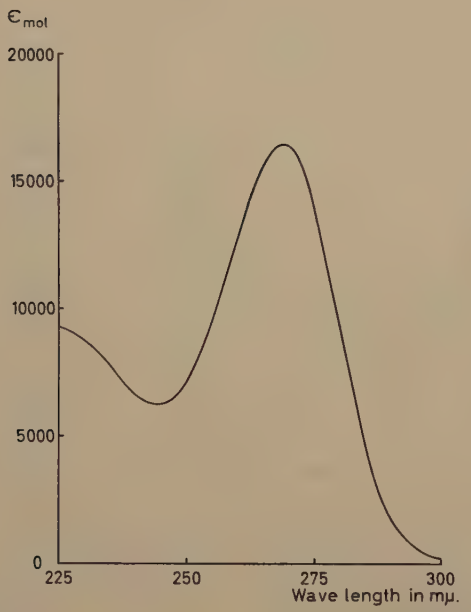

Fig. 1. Molar U.V.-absorption of PTH-valine in ethanol ($d = 1$ cm).

gentle evaporation in a desiccator over potassium hydroxide. A few drops of water were added and the evaporation procedure repeated twice in this way. The dry residue was dissolved in a freshly prepared buffer (50 μl). Phenyl *iso*-thiocyanate (6 μl) was dissolved in freshly distilled acetone (1 ml) and 50 μl of this solution added to the buffer solution. The phenyl *iso*thiocyanate should be present in at least a twofold molar excess over the amino acids. The tube was gently shaken to ensure complete mixing, well covered, and placed in a water bath at 25°C for 2 $\frac{1}{2}$ hours. It was then placed in a desiccator over phosphorous pentoxide and the content carefully evaporated for 15 min. using a water pump and finally for 2 hours in a vacuum of at least 0.01 mm Hg (cold trap!). This operation removed every trace of phenyl *iso*thiocyanate, triethylamine and acetic acid. A blank without amino acid was treated in the same way.

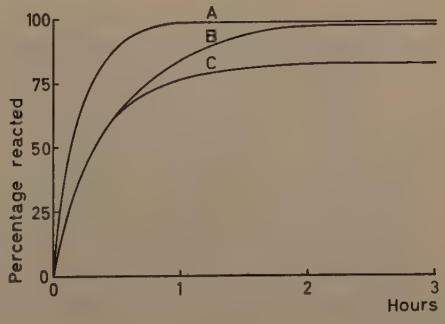

Fig. 2. PTC-coupling *v.* time. Valine (A), aspartic acid (B), serine (C). Temperature 25°C, pH 10.1.

Table 1. Molar extinction coefficients of various PTH's

PTH-derivative of	$\varepsilon_{245\ m\mu}$	$\varepsilon_{269\ m\mu}$	$\varepsilon_{245}/\varepsilon_{269}$
alanine	6,300	16,000	0.39
arginine[a]	6,000	15,900	0.38
aspartic acid	6,500	16,100	0.40
cysteic acid[b]	5,900	16,600	0.36
glutamic acid	6,200	15,900	0.39
glycine	6,300	14,900	0.42
histidine[a]	6,400	15,500	0.42
hydroxyproline	8,900	13,600	0.65
isoleucine	6,500	17,000	0.38
leucine	6,000	16,700	0.38
lysine[c]	21,000	29,000	0.72
methionine	6,900	17,100	0.40
phenylalanine	5,700	15,500	0.37
proline	9,600	14,300	0.67
serine	5,500	15,500	0.35
threonine	7,200	15,600	0.46
tryptophan	8,000	19,700	0.41
tyrosine	6,600	15,600	0.42
valine	6,200	16,500	0.38

[a] monohydrochloride.
[b] potassium salt.
[c] ε-phenyl thioureido-.

The effect of variations of reaction time, pH, temperature, and excess of phenyl *iso*thiocyanate was studied. The yield was measured by subjecting the dried reaction products to the conditions of stage II (cf. Sec. C) and measuring the yield in U.V.-absorption at 269 mμ. With regard to the duration of treatment, it was found that most of the amino acids reacted quantitatively with phenyl *iso*thiocyanate in 1 hour, the notable exceptions being aspartic acid, glutamic acid, and serine, where the reaction required a longer time to reach completion (Fig. 2). An increase in the concentration of phenyl *iso*thiocyanate did not appreciably increase the rate of reaction but tended to give a higher blank, presumably caused by nonvolatile hydrolysis products of the phenyl *iso*thiocyanate. As was expected, higher pH and temperature accelerated the rate of reaction. However, no particular advantage was found in modifying the chosen conditions.

C. *Transformation of PTC-amino acids into PTH-amino acids (Stage II)*

To the dry residue resulting from stage I 100 μl of water and 200 μl of acetic acid-hydrogen chloride were added. The tube was well covered and left in a water bath at 25°C for 6 hours. Afterwards, the solvent was evaporated in a desiccator over potassium hydroxide in the way described in the preceding section. The dried sample was dissolved in a suitable volume of ethanol for the measurement of U.V.-absorption.

The synthesis of every individual PTH-amino acid was checked by paper chromatography in several solvent systems. In no instance did this test reveal any heterogeneity of the product.

No study of the reaction rate of the individual PTC-amino acids was done, but the indication was that PTC-glycine cyclisized at lowest rate (Figs. 3 and 4).

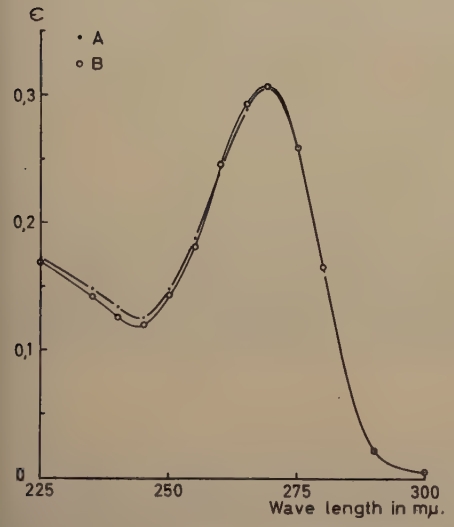

Fig. 3. U.V.-absorption during cyclisation of PTC-valine after 1 h (A), and 3 h (B). Temperature 25°C.

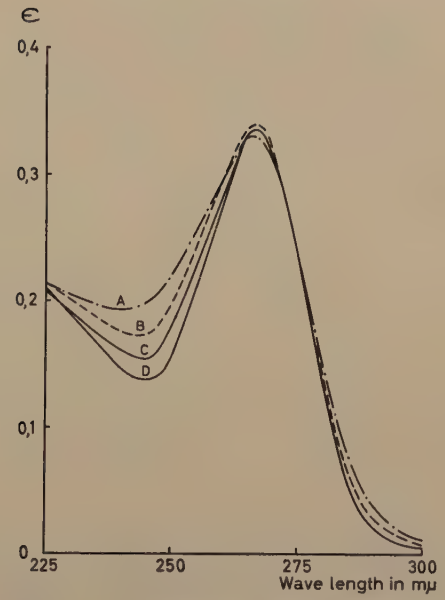

Fig. 4. U.V.-absorption during cyclisation of PTC-glycine after 1 h (A), 2 h (B), 3 h (C), and 5 h (D). Temperature 25°C.

The results are summarized in Table 2.

Table 2. The yields of PTH's in the micro synthetic procedure.

PTH-derivative of	Yield in p.c.	$\varepsilon_{245}/\varepsilon_{269}$
alanine	95.5	0.40
arginine	101.8	0.39
aspartic acid	97.5	0.41
cysteic acid[a]	108.4	0.40
glutamic acid	97.3	0.41
glycine	98.2	0.41
histidine	95.4	0.46
hydroxyproline	99.8	0.64
isoleucine	98.6	0.39
leucine	98.6	0.38
lysine[b]	96.4	0.72
methionine	99.0	0.39
phenylalanine	99.4	0.39
proline	96.4	0.66
serine	82.6	0.50
threonine	98.5	0.47
tryptophan	98.9	0.44
tyrosine	99.7	0.43
valine	98.9	0.39

[a] The free acid.
[b] ε-phenyl thioureido-.

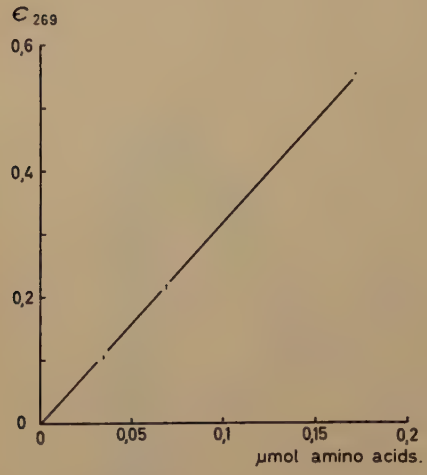

Fig. 5. U.V.-absorption of PTH's at 269 mμ *v.* quantity of amino acid mixture used for micro synthesis.

The yield is close to 100 p.c. in most instances. Considering that the yield refers to two reactions, stage I and stage II, it seems reasonably satisfactory. The most notable exception is serine with a deficit of 18 p.c. The cause of this deficit is known to be a side reaction involving a dehydration of the side chain of serine (4). The dehydration product also makes itself noticeable in an extra absorption peak at 320 mμ (7). The tendency of threonine to undergo an analogous reaction is much less and is hardly noticeable under the chosen conditions. It is relevant for the analytical procedure, that the deficit in PTH-serine is remarkably constant and hardly affected by the duration of treatment in stage II.

The ratio $\varepsilon_{245}/\varepsilon_{269}$ serves as a sensitive criterion of the purity of the products obtained. A comparison of Table I and Table II shows a generally satisfactory agreement of the ratio $\varepsilon_{245}/\varepsilon_{269}$ for the authentic PTH's and for the microsynthetic products. However, PTH-serine again forms an exception and probably for the reasons already discussed.

The quantity of amino acids was varied over a wide range and the overall yield of PTH's in the micro synthesis determined. A mixture of 18 amino acids[1] in approximately equimolar amounts was used, and varying amounts of this were subjected to the procedure described in Sec. B and Sec. C. When the extinction at 269 mμ was plotted against the quantity of amino acids, a strict proportionality was obtained over the whole range (Fig. 5).

SUMMARY

The use of 3-phenyl-2-thiohydantoins in amino acid analysis has been explored. A procedure for the quantitative synthesis of phenyl thiohydantoins from microgram quantities of amino acids is described. The procedure takes advantage of the high ultraviolet absorption displayed by these derivatives.

[1] The amino acids included in Table 2 with the exception of cysteic acid.

Thanks are due to Dr. Pehr Edman for valuable suggestions and discussions during the course of this work. The technical assistance of my wife, Mrs. Inga Sjöquist, is gratefully acknowledged.

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ARKIV FÖR KEMI Band 11 nr 15

Communicated 10 April 1957 by Hugo Theorell and Erik Jorpes

On the metabolism of bishomocholic, homocholic and norcholic acids in the rat

Bile acids and steroids. 50

By Sven Lindstedt and Nils Tryding

With 8 figures in the text

When bile acids are formed from cholesterol *in vivo*, the side chain is shortened by three carbon atoms. It has been suggested that the degradation starts with a methyloxidation (1), which is then followed by a β-oxidation. If this is the case, bile acids with 27 carbon atoms would appear as physiological intermediates, and such acids have in fact been found in Nature (see Haslewood, (2)).

In order to gain some information on the metabolism of bile acids with different length of the side chain, we have administered tritium-labelled bishomocholic, homocholic and norcholic acids to rats and studied their metabolites in the bile.

Experimental

Bishomocholic acid was prepared by anodic cross coupling of methyl hydrogen succinate with tritium-labelled cholic acid (3). This had been prepared by platinum-catalyzed exchange in tritium water (4) at an alkaline pH and purified by chromatography (phase system C). The bishomocholic acid obtained was purified by chromatography (phase system F) and crystallized from ethyl acetate. M.p.: 191–93°. Specific activity: 8.8×10^6 c.p.m./mg when counted in an infinitely thin layer in a "Tracerlab" gas-flow counter. (With this equipment 1 μC is equivalent to $\sim 8.3 \times 10^5$ c.p.m.)

Homocholic acid was prepared by anodic cross coupling of methyl hydrogen malonate with the tritium-labelled cholic acid. It was purified by chromatography with phase system C. M.p. 216°. Spec. activ.: 1.21×10^5 c.p.m./mg.

Norcholic acid was prepared by a Barbier–Wieland degradation of cholic acid. The inactive product (17 mg) was left with 1 C of tritium gas for 5 days (5). After dilution with unlabelled acid (25 mg), the material was chromatographed with phase system C. A small band appearing before norcholic acid indicated that some degradation products of a more polar nature had been formed. The most active fractions of the norcholic acid band were combined and crystallized from ethyl acetate. M.p. 189–90°. Spec. activ.: 7.4×10^5 c.p.m./mg.

1–2 mg of the acids was dissolved in a few drops of ethanol and neutralized and diluted to approximately 2 ml with saline. This solution of the sodium salt was

then given by intraperitoneal injection to bile fistula rats. The bile was collected into ethanol. After filtering off the precipitated proteins, the bile acids were extracted from the acidified solution with *n*-butanol. Saponifications of the conjugated acids were carried out with 2 *N* sodium hydroxide in sealed glass tubes at 120°C for 8–12 hours.

Chromatographic separations of the bile acids were performed by the methods developed by Bergström, Sjövall and Norman (6, 7, 8). The following phase systems were used:

	Moving phase	Stationary phase
F.	Methanol/water 55/45	Chloroform/heptane 9/1
C.	Methanol/water 50/50	*Iso*-octanol/chloroform 1/1
D.	Water	*n*-Butanol

The supporting medium was hydrophobic kieselguhr. Each fraction was titrated with 0.02 *N* sodium hydroxide. A suitable aliquot was plated on a copper or aluminium planchet and counted in a window-less flow-counter. Owing to the low energy of the β-radiation of tritium, self-absorption and irregularities in the geometry play a large role, and the results are therefore more of a qualitative than of a quantitative nature.

Results

Fig. 1 shows a chromatogram of the unhydrolyzed bile of a rat that had recieved 0.5 mg of labelled *bishomocholic acid*. One large peak of labelled material is seen at 15–25 ml. It appears at the position of taurocholic acid, which is the main bile acid

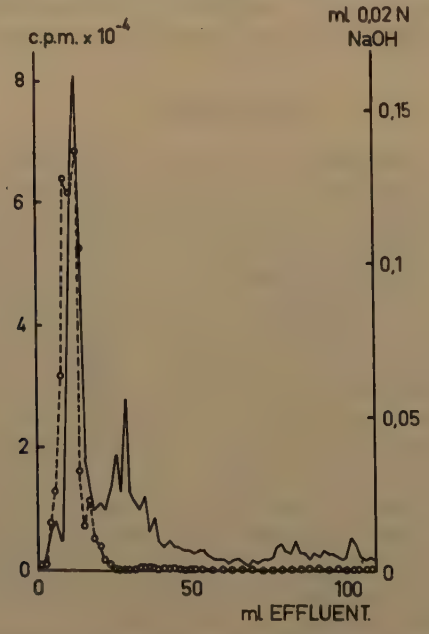

Fig. 1. Conjugated bile acids after intraperitoneal administration of labelled bishomocholic acid. Phase system D. Broken line: Radioactivity. Solid line: Titration.

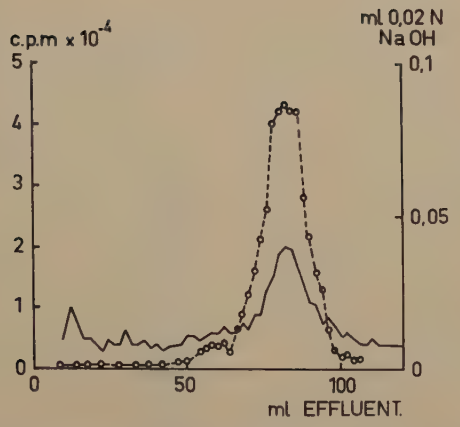

Fig. 2. Identification of cholic acid after hydrolysis. Phase system C. Broken line: Radioactivity. Solid line: Titration.

in rat bile. The saponified acids were extracted and chromatographed with phase system C, which is suitable for the isolation of cholic acid. In Fig. 2 the coincidence of the titration peak with the radioactivity peak is apparent. For further identification the material was diluted with 200 mg of cholic acid and crystallized from EtOAc, EtOH, acetone/water, EtOAc. The specific activity was determined by gas-counting (9) and found to remain constant. About 3 per cent of the activity put onto the column was retained in the stationary phase. It appeared in the front when run with phase system C.

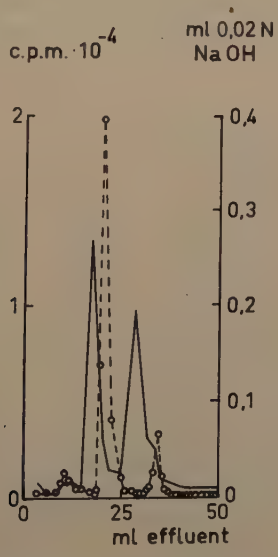

Fig. 3. Conjugated bile acids after intraperitoneal administration of labelled homocholic acid. Phase system D. Broken line: Radioactivity. Solid line: Titration.

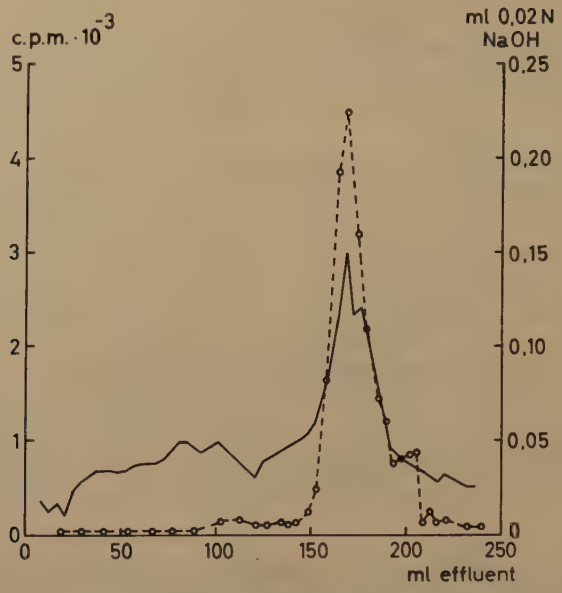

Fig. 4. Identification of homocholic acid after hydrolysis. Phase system C. Broken line: Radioactivity. Solid line: Titration.

Homocholic acid.—Fig. 3 shows a chromatogram of the conjugated bile acid obtained from bile during 24 hours after the administration of labelled homocholic acid (0.5 mg). The main radioactive band appears at 20–25 ml just behind the taurocholic acid. After hydrolysis this band was run with carrier homocholic acid with phase system C. The resulting chromatogram is seen in Fig. 4. In Fig. 3 a small

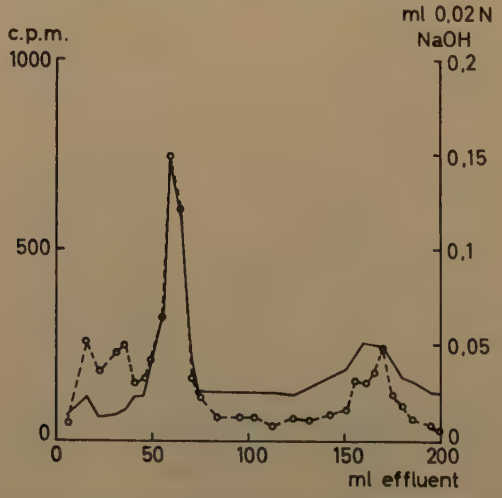

Fig. 5. Identification of free norcholic and homocholic acid in bile. Phase system C. Broken line: Radioactivity. Solid line: Titration.

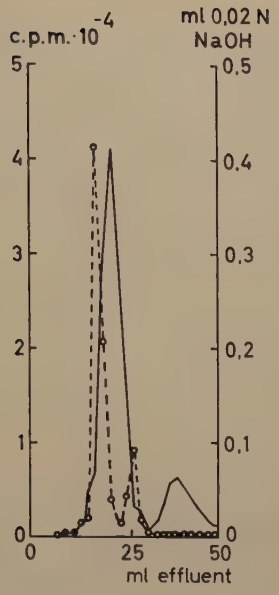

Fig. 6. Conjugated bile acids after intraperitoneal administration of labelled norcholic acid. Phase system D. Broken line: Radioactivity. Solid line: Titration.

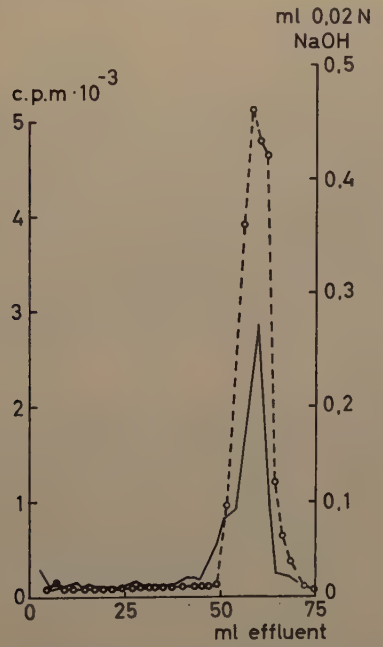

Fig. 7. Identification of norcholic acid after hydrolysis. Phase system C. Broken line: Radioactivity. Solid line: Titration.

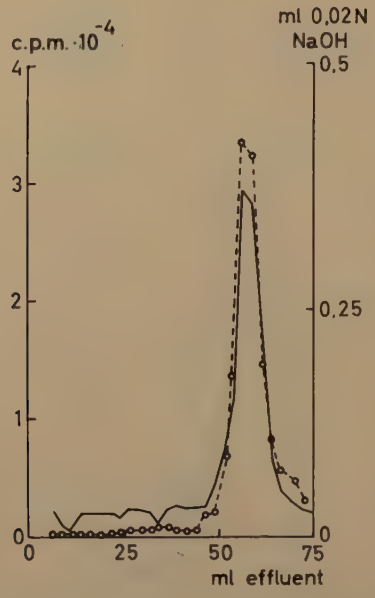

Fig. 8. Identification of free norcholic acid excreted in bile. Phase system C. Broken line: Radioactivity. Solid line: Titration.

active band is seen at 15–20 ml. It appears at the position to be expected for a taurine conjugated norcholic acid. Hydrolysis and chromatography with carrier norcholic acid indicated the identity with this acid. About 10 per cent of the activity put onto the column in Fig. 3 remained in the stationary phase. After elution it was run with phase system C together with inactive norcholic and homocholic acids. Fig. 5 shows that labelled material was eluted at the position of norcholic acid (50–75 ml) and of homocholic acid (150–200 ml). The norcholic acid was recrystallized three times from EtOAc. The specific activity remained constant. In none of the four animals studied did the amount of norcholic acid formed exceed 10 per cent of the recovered activity.

Norcholic acid.—0.5 mg of norcholic acid was administered to a rat and the conjugated bile acids fractionated with phase system D. Fig. 6 shows that the main labelled band appeared at 10–20 ml. This material was hydrolyzed and rerun with carrier norcholic acid in phase system C. The identity of the labelled material with norcholic acid is obvious from Fig. 7. In the experiment shown in Fig. 6 about 80 per cent of the activity remained on the column. After elution the material was rerun with phase system C with unlabelled norcholic acid, when the result seen in Fig. 8 was obtained. In one experiment the unhydrolyzed bile was directly chromatographed with phase system C. It was found that about 5 per cent of the activity appeared in the front (taurine conjugates), the remainder being located at the position of free norcholic acid.

Discussion

Bishomocholic acid was efficiently degraded to cholic acid, which was then not further attacked. The metabolism of 3α,7α,12α-trihydroxycoprostanic acid has earlier been studied. This acid which differs from bishomocholic acid by the presence of a methyl group in α-position to the carboxyl group was rapidly degraded to cholic acid by the rat liver *in vivo* (10) or *in vitro* (11). In these experiments no evidence was obtained for the further degradation of the cholic acid formed. The similar metabolic pattern of the two acids was to be expected in analogy with conditions in the fatty acid series, where straight chain and α-monomethyl-branched acids show a similar susceptibility to β-oxidation (12).

Bile acids with less than 24 carbon atoms have not been found in Nature, and the β-oxidation of this structure seems to be hindered. Although an α-β-dehydrogenation should be formally possible, the formation of an appropriate enzyme-substrate complex is evidently hindered by the proximity of the steroid nucleus.

When *homocholic acid* was given it was possible to isolate a small percentage of the recovered activity in the form of norcholic acid, thus demonstrating the gradual increase in the hindering effect of the steroid nucleus on side chain degradation.

In studies of the metabolism of branched chain fatty acids the blocking effect of chain substituents has earlier been encountered (13). It was recently found that the degradation of 2,2-dimethylstearic acid from the ω-end mainly stopped at the 2,2-dimethyladipic acid stage (14). When on the other hand 2,2-dimethylnonadecanoic acid was the starting material it was found that the β-oxidation could reach one step nearer to the blocking residue, and 2,2-dimethylglutaric acid was isolated as the main metabolic end-product (15).

The results obtained support the view that the degradation of the cholesterol side chain to that of a bile acid proceeds through a methyl oxidation followed by one β-oxidation (1). When on the other hand cholesterol acts as a precursor of progesterone a completely different mechanism must be operating as is also evident from the work by Lynn, Staple and Gurin who found that in adrenal homgenates the side chain is split off as isocaproic acid (16, 17).

Conjugation.—In several studies on the metabolism of different C-24 bile acids in the rat they have been found to be excreted in the bile conjugated with taurine and to a small extent with glycine. Free bile acids in the bile have never been encountered (cf. Norman, (18)). In accordance with these results the cholic acid formed from bishomocholic acid appeared in the bile as its taurine conjugate.

Homocholic and norcholic acids also gave rise to conjugated acids. The chromatographic mobility suggested that they were conjugated with taurine. Norcholic as well as homocholic acid was, however, also excreted in the free form in the bile. Homocholic acid was found to be conjugated to more than 95 per cent, while norcholic acid was only conjugated to 5–10 per cent. The inefficiency of the conjugation mechanism when norcholic acid was the substrate might be explained in a similar way as for the β-oxidation. It is known that the primary step in the conjugation is the formation of a bile acid CoA compound (19, 20, 11), the formation of which might be hindered by the proximity of the steroid nucleus.

SUMMARY

Bishomocholic, homocholic and norcholic acids have been prepared and labelled with tritium. Their metabolites in the bile of fistula rats have been studied.

1. Bishomocholic acid was completely degraded to cholic acid.

2. Homocholic acid was degraded to norcholic acid to less than 10 per cent. A small percentage of the homocholic acid was also found in the bile as uncounjugated acid.

3. Norcholic acid was excreted in the bile mostly in the free form. Less than 20 per cent was excreted conjugated with taurine.

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The technical assistance of Mr. Bengt Åkesson is gratefully acknowledged.

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Tryckt den 11 april 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 16

Communicated 10 April 1957 by Hugo Theorell and Erik Jorpes

The formation of deoxycholic acid from cholic acid in man

Bile acids and steroids. 52

By Sven Lindstedt

With 5 figures in the text

During recent years the metabolism of different bile acids has been extensively studied in experimental animals (for reviews see (1, 2)). However, the fate of these compounds in man is largely unknown.

In this paper evidence for the formation of deoxycholic acid from cholic acid is presented.

Experimental

The technique of feeding labelled cholic acid and the isolation of the free bile acids from human bile has been described earlier (3).

Feces from the experimental subjects were collected in ethanol and the bile acids extracted as described by Lindstedt and Norman (4).

Chromatographic separations were carried out with the reversed phase technique as described by Bergström, Sjövall and Norman (5, 6, 7). The phase systems are given in the figure legends.

The radioactivity was determined in a "Tracerlab" gas-flow counter. A direct plating technique was used. Owing to the restricted amount of labelled cholic acid that could be used in human experiments the specific activity of the fecal material was low, and self-absorption was considerable. The quantitative aspects have, however, not been stressed in this exploratory work.

Results

The isolation of labelled deoxycholic acid from bile after administration of cholic acid-24-^{14}C.

The bile samples examined were obtained from subjects that had recieved $\sim 3\ \mu C$ of cholic acid-24-^{14}C during a study of the turnover of cholic acid in man. After the isolation of the cholic acid from the hydrolyzed bile the less hydrophilic acids were chromatographed. Figs. 1 and 2 show the chromatograms obtained from the bile of two subjects (L. P. and E. H.) three days after the administration of the labelled acid. In both cases there is a titration peak at 50–80 ml that coincides with a peak of radioactivity.

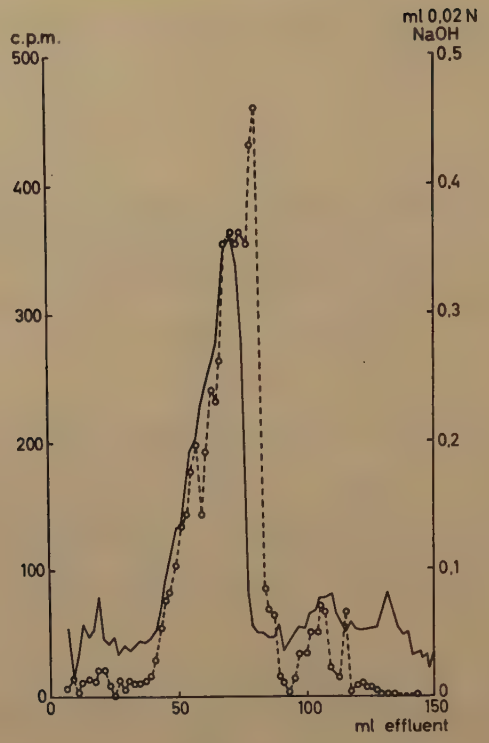

Fig. 1. Dihydroxycholanic acids in the bile of subject L. P. three days after the administration of cholic acid-24-^{14}C. Phase system F. Moving phase: Methanol/water 55/45. Stationary phase: chloroform/heptane 9/1. Broken line: Radioactivity. Solid line: Titration.

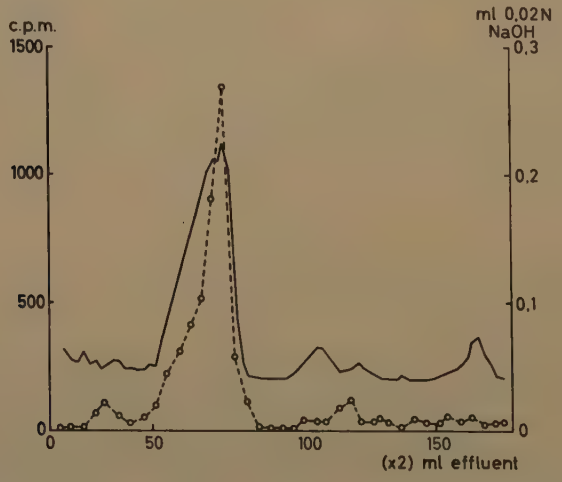

Fig. 2. Dihydroxycholanic acids in the bile of subject E. H. three days after the administration of cholic acid-24-^{14}C. Phase system F. Broken line: Radiactivity. Solid line: Titration.

Table 1. Recrystallization of deoxycholic acid from subject L. P.

Sample	Weight, mg	c.p.m./mg	Solvent
L. P. 3, 1st cryst.	19.0	1600	HAc/aq.
L. P. 3, 2nd cryst.	10.7	1800	HAC
L. P. 3, 3rd cryst.	8.8	1850	HAc/aq.
L. P. 3, 4th cryst.	3.8	2000	EtOAc/light petroleum
L. P. 5, 1st cryst.	19.5	830	HAc/aq.
L. P. 5, 2nd cryst.	9.0	1100	HAc
L. P. 5, 3rd cryst.	4.5	1300	HAc/aq.
L. P. 5, 4th cryst.	1.3	1300	EtOAc/light petroleum

The material contained in the labelled band shown in Fig. 1 at 50–90 ml was collected and the free acids extracted with ether after acidification. In the same way the dihydroxy-acids were isolated from the bile of the same subject 5 days after the administration of the labelled cholic acid. A series of crystallizations was carried out and the specific activity determined. The data are summarized in Table 1. The last crystallization gave material with the m.p. 169–171°C after drying *in vacuo* at 111°C for 24 hours. Paper chromatography according to Sjövall (8) gave only one spot at the position of deoxycholic acid. In the system used chenodeoxycholic and deoxycholic acids are separated. Finally the methyl ester of sample L. P. 3 was prepared with diazomethane, m.p. 103–107.

Identification of labelled free deoxycholic acid in the feces after the administration of cholic acid-24-^{14}C.

Feces collected on the second and fourth day after the administration of labelled cholic acid to a young man were studied. Sample A. W. 2 was first run with a phase system where the dihydroxycholanic acids are retained in the column. In the chromatogram (Fig. 3) a labelled band at the position of free cholic acid (80–110 ml) can be seen. The material retained in the column was eluted and rerun with a phase system suitable for the separation of less hydrophilic compounds. Fig. 4 shows the presence of several bands of labelled material. One of these appears at the position of a dihydroxycholanic acid (50–80 ml), where a titration peak is also to be noticed. The activity of this peak constituted about ten per cent of the total activity in the fecal extract. The material in the band was rechromatographed with the same phase system for further purification. Paper chromatography according to Sjövall (8) showed that both chenodeoxy- and deoxycholic acids were present. When the deoxycholic acid spot was eluted and counted it was, however, found to contain at least 80 per cent of the activity put onto the chromatogram. 25 mg of inactive, deoxycholic acid was therefore added to the dihydroxy acid band. Crystallization was carried out twice from acetic acid/water and twice from ethyl acetate/light petroleum. The specific activity was found to remain essentially constant.

Sample A. W. 4 was directly chromatographed with the phase system suited for the isolation of less hydrophilic acids (Fig. 5). A large titration peak that contained about 30 per cent of the material put onto the column was eluted at 0–30 ml. A band of labelled material was found at the position of the dihydroxycholanic acids. It accounted for about ten per cent of the total activity in the fecal extract. The

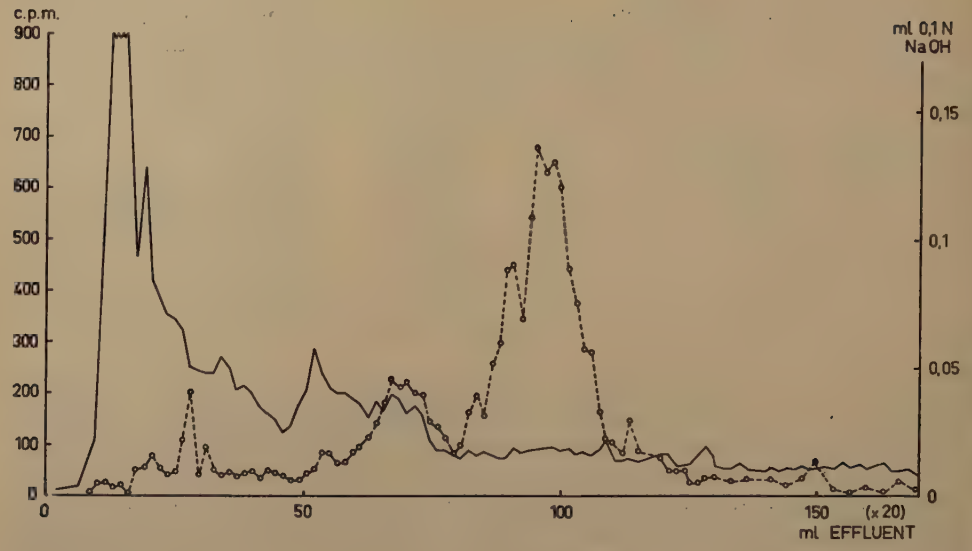

Fig. 3. Fecal bile acids from subject A. W. on the second day after administration of cholic acid-24-^{14}C. Phase system C. Moving phase: methanol/water 50/50. Stationary phase: *iso*-octanol/chloroform 50/50. Broken line: Radioactivity. Solid line: Titration.

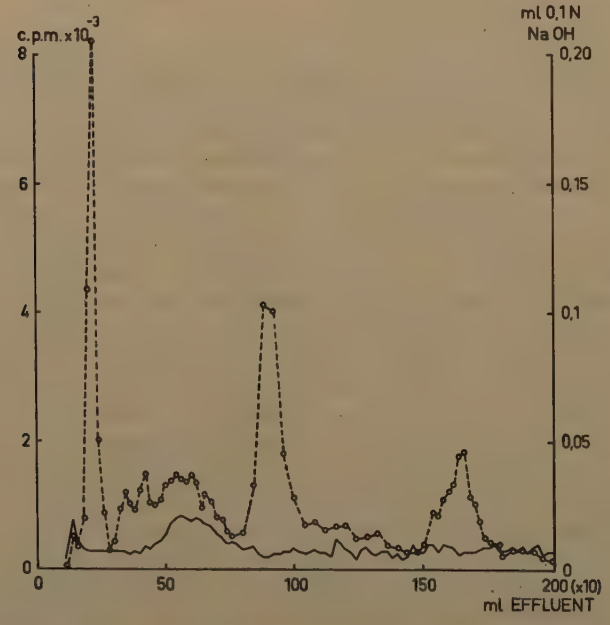

Fig. 4. Fecal bile acids retained and eluted from the column of Fig. 3. Phase system F. Broken line: Radioactivity. Solid line: Titration.

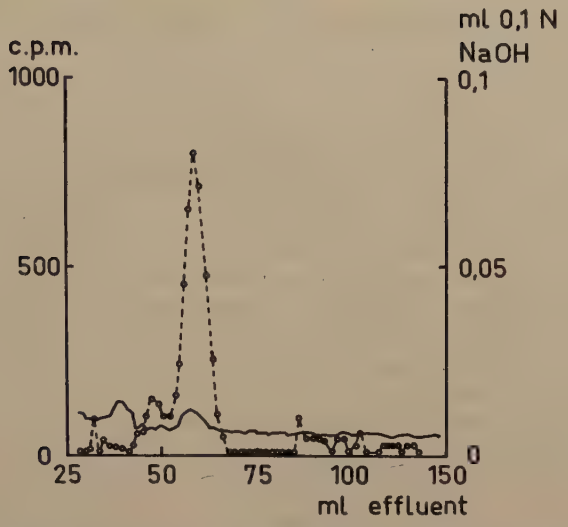

Fig. 5. Fecal bile acids from subject A. W. on the fourth day after administration of cholic acid-24-^{14}C. Phase system F. Broken line: Radioactivity. Solid line: Titration.

acids were diluted with carrier deoxycholic acid and recrystallized as above. The specific activity remained constant during this procedure. Fecal extracts from two other subjects that had recieved labelled cholic acid have also been chromatographed. A band of labelled material was eluted at the position of the dihydroxy-acids in both cases.

Discussion

It has recently been found that cholic acid gives rise to deoxycholic acid in the rabbit when it is allowed to take part in an intact enterohepatic circulation for three days (9). When on the other hand labelled cholic acid was given to animals with a bile fistula no formation of deoxycholic acid was observed. This was taken to indicate that the intestinal microorganisms were responsible for the formation of the deoxycholic acid in the rabbit. Sjövall (10) has found that deoxycholic acid is absent from human bile collected from surgical fistulas. Furthermore, the bile of newborn babies does not contain any deoxycholic acid (11).

The important question in this connection is whether deoxycholic acid is only formed secondarily from cholic acid or whether it might arise from the degradation of cholesterol in the liver to some extent. Ekdahl and Sjövall (12) isolated labelled deoxycholic acid from the bile after the administration of cholesterol-4-^{14}C to a rabbit. In this experiment the cholesterol was given 3 days before the bile fistula was made and the action of the intestinal microorganisms was thus not excluded. It has been claimed that labelled deoxycholic acid is excreted in the fistula bile of rat (13) and man (14) after the administration of labelled cholesterol. It seems possible, however, that the contamination with labelled cholic or chenodeoxycholic acid was not rigorously excluded in these experiments.

Some experimental evidence (15, 16) supports the view that a primary step in the

elaboration of the bile acid structure *in vivo* is a hydroxylation in the 7-position of cholesterol.

If this is a general reaction chenodeoxy- and cholic acids would be the "primary" bile acids, while deoxycholic acid in human bile might originate from the secondary action of intestinal microorganisms on these compounds.

The excretion of labelled acidic material in the feces of rats and rabbits fed cholesterol (17) or bile acids (4) has been studied. In these investigations a rich variety of compounds was found. When cholic acid was given much of the radioactivity was found in material of a less hydrophilic nature, and it was observed that a labelled band appeared at the position of a dihydroxycholanic acid. In view of these investigations it was not surprising to find that the labelled cholic acid given to humans was extensively attacked during the intestinal passage. Carey and Watson (18) have isolated free deoxycholic acid from human feces and are of the opinion that this is the main bile acid excreted. As, however, only about 10 per cent of the excreted activity was found in the deoxycholic acid in the samples examined in this work, the question seems to require further investigation.

SUMMARY

After administration of ^{14}C-labelled cholic acid to humans the deoxycholic acid isolated from bile contained radioactivity. In the feces a variety of labelled compounds was found, among which free deoxycholic acid was identified.

This work is part of investigations supported by "Knut och Alice Wallenbergs Stiftelse", Stockholm, and the Medical Faculty, Lund.

The technical assistance of Miss K. Hyllén and Mr. U. G. Lindeberg is gratefully acknowledged.

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Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 17

Communicated 10 April 1957 by Hugo Theorell and Erik Jorpes

Determination of amino acids as phenyl thiohydantoin derivatives

II. Chromatographic separation of 3-phenyl-2-thiohydantoins of amino acids

By John Sjöquist

With 5 figures in the text

In the first paper of this series (1) a microsynthetic procedure for the quantitative conversion of amino acids into phenyl thiohydantoin (PTH) derivatives was described.

In this report column and paper chromatographic procedures for the separation of PTH's in the microsynthetic procedure are described. In conjunction, these operations form the basis of a micro analytical determination of amino acids.

Successful separation of the PTH's by means of partition chromatographic procedures have been reported earlier. Thus, Sjöquist (2), and Landmann, Drake, Dillaha (3) were able to separate these derivatives by paper chromatography and improvements have later been published (4). Furthermore Rovery, Fabre, and Desnuelle (5) have described a column chromatographic separation of the PTH-derivatives of the amino acids alanine and isoleucine. From this laboratory a column chromatographic separation of PTH's with provisions for the automatic registration of the separated peaks by means of their strong U.V.-absorption has been preliminarily reported (6). This work has now been considerably extended aiming at the complete analysis of the amino acids normally constituting a protein hydrolysate.

This goal has largely, though not completely, been reached. It has not been possible as yet to find one solvent system permitting resolution of the eighteen zones by a single chromatographic operation. However, the use of three different systems allows quantitative determination of sixteen PTH-derivatives.

Experimental

A. *Equipment, materials and methods*

Equipment.—The chromatographic tube (Fig. 1) is made completely of glass and is provided with a water jacket for thermostatic control of the content. Water from a thermostat (25°C) is circulating through the jacket. The connections to the liquid pump and to the light absorption cell are established by spherical joints. To the top of the column a tube for holding an air cushion is connected, which serves to even out the pressure of the discontinuous pump.

The arrangements for the automatic registration of the U.V.-absorption in the effluent (7) as well as the precision liquid pump (8) have been described earlier.

The perfect fit of the joints between the different parts of the apparatus makes grease unnecessary.

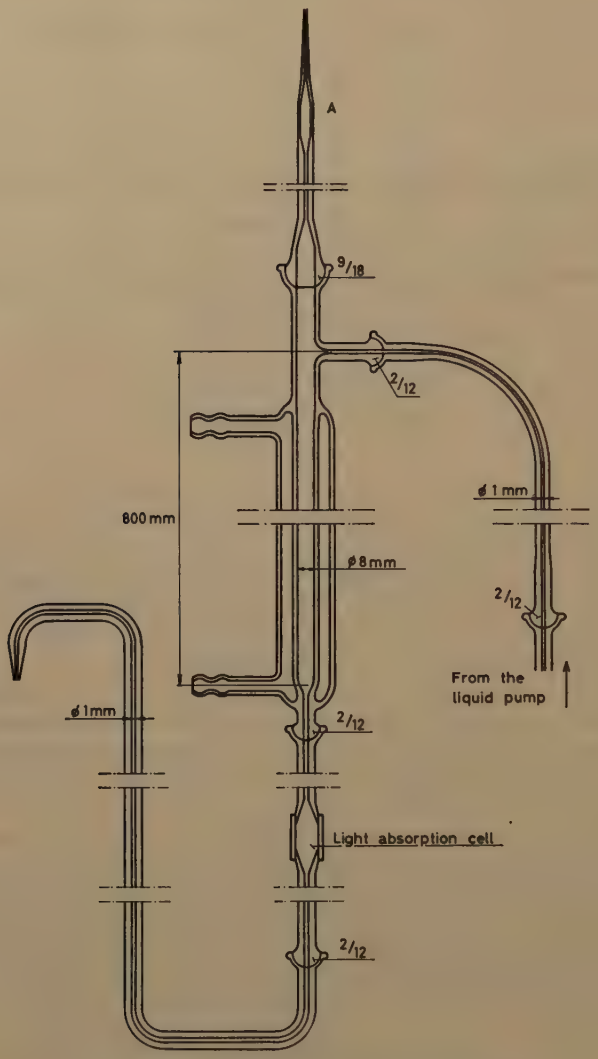

Fig. 1. The chromatography tube with parts attached.

Materials.—*n*-Butanol, Analytical reagent (Baker).

Formic acid 98–100%, pro analysi (Merck). Diluted with water to 75% v/v.

n-Heptane (Eastman Kodak) is stored over conc. H_2SO_4 for 3 weeks, and then washed repeatedly and successively with water, 10% aqueous NaOH and water. It is finally dried over anhydrous Na_2SO_4. It is distilled, and the fraction boiling 98–99°C is collected.

Ethylene chloride, puriss (Fluka) is shaken three times with $\frac{1}{10}$ its vol. of conc. H_2SO_4, washed successively with water and 5% aqueous Na_2CO_3, and then dried over K_2CO_3. It is finally distilled and fractions boiling at 83.5°C are collected.

Propionic acid (Eastman Kodak) is refluxed for 3 hours with chromic acid (Merck *pro analysi*, 25 g/750 ml. *Cave* violent reaction!). After cooling the propionic acid is poured off, distilled on a Widmer column and the fraction boiling at 140–141°C is collected.

Glass-distilled water is used throughout.

With the exception of *n*-butanol and formic acid the U.V. absorption of the solvents at 269 mμ should be nil or negligible.

Celite, Analytical Filter-Aid (Johns-Manville) is further purified by storage over conc. HCl for 3 days, washing first with water until pH has risen to ~4 and then twice with a 0.1% aqueous ethylene diaminotetraacetic acid. It is subsequently washed with water until the washings are neutral and finally with a good grade 95% ethanol. The preparation is dried in an oven at 100°C and kept there until immediately before use.

The bovine serum albumine is the commercial product of the Armour Laboratories. The protein nitrogen content of the solution is 9.9 mg/ml.

The amino acids used are those specified in a preceding publication (1).

Methods.—All measurements of light absorption are made in matched quartz absorption cells (d = 1 cm) in a Beckman Spectrophotometer Model DU.

Paper chromatography is carried out as described by Edman and Sjöquist (4).

The micro synthesis of the PTH's from amino acids is carried out as described earlier (1).

B. *Procedure*

Preparation of column.—Ten grams of Celite is intimately mixed with 6 ml of the phase later to become the stationary. This mixture is then made into a slurry by thorough stirring with approximately 100 ml of the phase later to become the mobile.

A small cotton plug is inserted in the capillary at the bottom of the chromatography tube and the lower part of the tube is filled with the mobile phase. Air retained by the cotton is carefully removed by applying slight suction from below and by pressing with a glass rod from above. A funnel provided with a spherical joint (9/18) is attached to the top of the column and the Celite slurry is added at once. The column is then packed with a stamp made of glass. This operation takes about 45 minutes. With a diameter of 0.8 cm the resulting column should have a height of approximately 50 cm. The top of the column is covered with a well fitting piece of filter paper.

Addition of the sample to the column.—The dry sample in the quartz tube in which the microsynthesis has been carried out is dissolved in 0.1 ml stationary phase and transferred with a capillary pipette to the top of the column following drainage of excess solvent from the latter. When the sample has drained into the column it is followed by two 0.1 ml washings of the same solvent from the quartz tube and finally with three 0.2 ml washings of mobile phase. The space above the column is subsequently filled with the mobile phase.

Assembling of the parts.—The lower delivery tube is attached with clamps to the outlet tube of the light absorption cell and this part of the apparatus is filled with the mobile phase by suction applied from the inlet tube of the cell. The chromatography tube is then attached to the upper joint of the absorption cell and the delivery tube from the liquid pump is fitted to the inlet of the chromatography tube. The upper end of the chromatography tube holding the air cushion (Fig. 1, *A*) is drawn

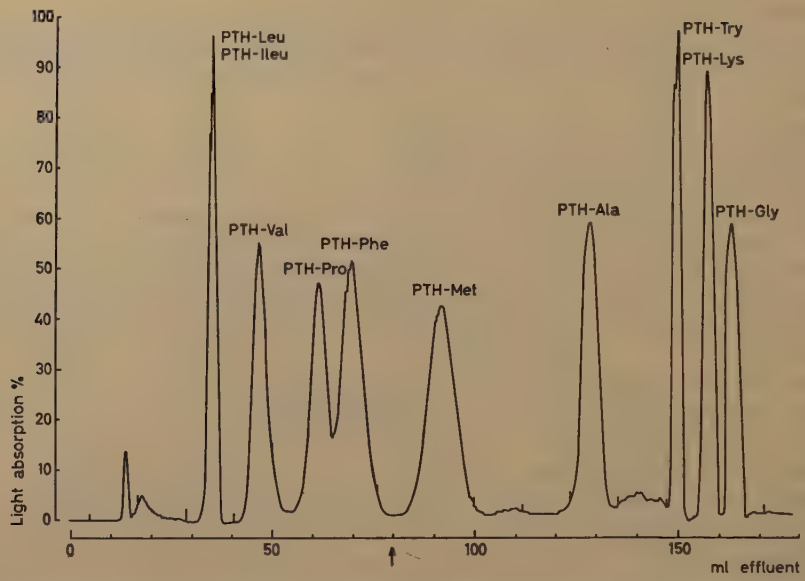

Fig. 2. Chromatographic separation of PTH-derivatives of amino acids on a Celite column. Automatic registration of the U.V. absorption at 269 mμ in the effluent. The arrow indicates change of solvent. Column I.

out into a capillary and this part put in place. The whole system is then filled with mobile phase using the pump but for a suitable volume in part *A*. The capillary tube at the top is then sealed off in a flame.

It is important, in order to avoid the formation of air bubbles in the absorption cell during the run, that the tip of the exit delivery tube is placed above the bottom level of the column. Furthermore, the upper valve of the pump should preferably be placed below the same level.

In the following the three different chromatographic operations for the separation of the PTH's are described which together form the analytical procedure. Two of the operations are carried out on columns, here called Column I and Column II, and these are supplemented by a paper chromatographic separation of PTH-arginine and PTH-histidine.

Column I.—The preparation of the column is described above.

The following two solvent systems are used:

A. Heptane, 250 ml; propionic acid, 100 ml; ethylene chloride, 5 ml; water, 30 ml.

The lower layer (78 ml) is used as the stationary phase and the upper layer as the mobile phase.

B. Heptane, 85 ml; propionic acid, 5 ml; ethylene chloride, 10 ml.

Only one phase is obtained.

The liquid pump is set to a flow rate of 15.0 ml/hour. The elution is started with solvent A. Solvent B is introduced when the peak of PTH-phenylalanine has left the column. The shift of solvents is done by changing the contents in the reservoir of the pump.

The result of a typical experiment is presented in Fig. 2. In this experiment approximately 0.1 μmole of each amino acid was used for the microsynthesis with the

exception of the leucines and lysine where about 0.05 μmoles of each amino acid were taken. As can be seen the following PTH's emerge as single peaks without mixing with the adjacent peaks: PTH-valine, PTH-methionine, PTH-alanine, PTH-tryptophan, PTH-lysine, and PTH-glycine. With regard to PTH-proline and PTH-phenylalanine the peaks are almost but not completely separated. The PTH-leucines emerge as a single peak although a split at the top is quite often seen.

Column II.—The column is operated in the same way as column I up to the point where the PTH-phenylalanine has completely left the column. From now on a gradient elution is applied. The gradient is set up in the following way:

In the reservoir of the pump 150 ml of the solvent A is left. To the reservoir a solvent mixture is then added at the flow rate of the pump. This solvent consists of heptane, 58 ml; propionic acid, 17 ml; ethylene chloride, 25 ml.

The addition of the second solvent is conveniently done from a separatory funnel the tip of which has been drawn out to a capillary. Complete mixing is ensured by a stirring motor. The flow rate of the pump is set to 15.0 ml/hour through the whole experiment.

The result of a typical run is presented in Fig. 3. The amounts of amino acids used were approximately the same as described for Column I. If only the peaks emerging after PTH-alanine are considered it can be seen that PTH-hydroxyproline,[1] PTH-tyrosine, PTH-glutamic acid and PTH-threonine appear as single peaks without admixture. PTH-aspartic acid and PTH-serine are almost completely separated. However, PTH-tryptophan, PTH-lysine, and PTH-glycine appear as a joint peak.

Paper chromatography.—For the separation of PTH-arginine and PTH-histidine the following paper chromatographic procedure has been used. The sample is applied with a Carlsberg pipette to a Whatman No. 1 paper which is sprayed with a 25% solution of formic acid in acetone. The paper is then left at room temperature for $\frac{1}{2}$ hour for the acetone to evaporate. The chromatographic vessel is lined with filter paper soaked in a 25% aqueous solution of formic acid. Descending chromatography is then performed with a solvent mixture consisting of heptane (40 ml), *n*-butanol (30 ml), and 75% aqueous formic acid (9 ml). The paper strip is dried for 15 minutes at 100°C and the spots then located and cut out under the guidance of the fluorescent screen (4).

The cut out pieces of filter paper are each soaked in 2 ml of 70% aqueous ethanol for $\frac{1}{2}$ hour at room temperature. The U.V. absorption of the ethanolic solution is then read in a Beckman Spectrophotometer Model DU at 269 mμ using micro absorption cells (d = 1 cm). A blank correction is obtained by cutting out on an empty lane a piece of filter paper corresponding in size and level to the sample. The R_F-value for PTH-histidine is 0.37 and for PTH-arginine 0.46.

C. *Recoveries*

The calculations of the recoveries are based on the amount of amino acids weighed in for micro synthesis on the one hand and on the U.V. absorption at 269 mμ of each peak or spot on the other hand. The theoretical yield is calculated from the known

[1] It is interesting to note that, when this chromatographic procedure was applied to the PTH-hydroxyproline preparation described by Edman (10), two partly separated peaks instead of one were clearly discernible. The explanation for this is probably to be found in the presence of the *allo*-forms of the particular sample of hydroxyproline used for the synthesis of the PTH-derivative. This is also indicated by the low melting point found by Edman.

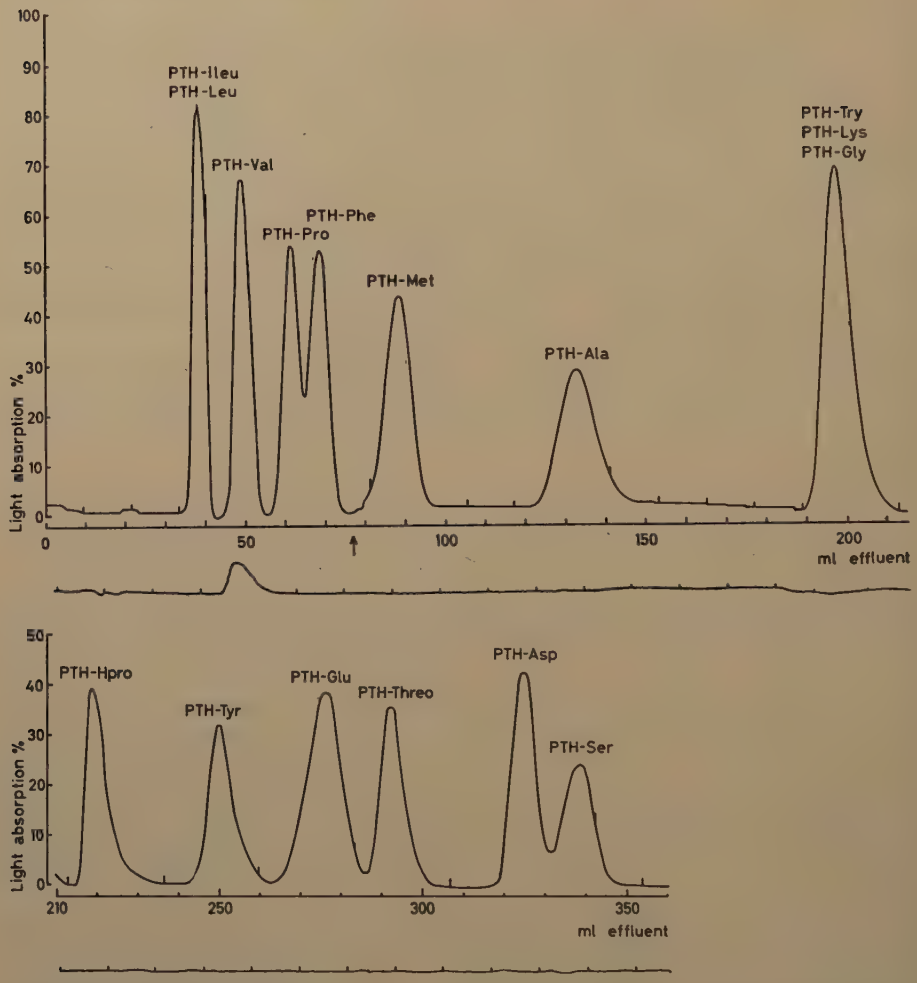

Fig. 3. Column II. The gradient is set in at the arrow. The lower curve is the tracing of a blank run without amino acids.

molar absorption coefficients of the various PTH's (1). The volume of effluent containing one peak is collected separately in a 50 ml round bottomed, long necked flask and the solvent afterwards continuously evaporated at room temperature *in vacuo*. In those instances, where two peaks are not completely separated (e.g. PTH-proline and PTH-phenylalanine, PTH-aspartic acid and PTH-serine) the cut is made at the lowest point of the valley between the peaks. The dried samples are dissolved in suitable volumes of spectrophotometrically pure ethanol and the U.V.-absorption at 269 mμ is measured in the usual way. With regard to blank correction for non volatile impurities in the chromatographic solvent this has been found to be so small as to be negligible. However, a correction of another kind enters in the case of PTH-valine. This is due to an impurity, apparently originating in the microsyn-

Table 1. Recoveries of PTH-amino acids separated by chromatography.

PTH-derivative of	Procedure	Recovery in per cent	
		1st run	2nd run
leucines	Column I or II	100.0	99.5
valine	„	100.0	99.5
proline	„	98.5	99.5
phenylalanine	„	98.0	98.0
methionine	„	100.5	100.0
alanine	„	98.5	100.0
tryptophan	Column I	97.5	99.0
lysine[a]	„	101.0	99.5
glycine	„	99.0	99.5
hydroxyproline	Column I or II	99.5	99.5
tyrosine	Column II	98.5	98.5
glutamic acid	„	99.5	97.5
threonine	„	71.5	72.5
aspartic acid	„	96.0	95.0
serine	„	41.5	40.0
arginine	Paper chromatography	95.0	99.5
histidine	„	95.5	97.5

[a] ε-phenyl thioureido-.

thesis, which travels with PTH-valine on the chromatograms (Fig. 3). The exact magnitude of this correction is difficult to establish without running chromatograms on a blank from the microsynthesis. However, it has been generally found that the U.V. absorption of this peak corresponds fairly well to the absorption of the blank in the microsynthesis. It has therefore been found convenient to subtract the latter blank from the absorption of the PTH-valine peak.

The recoveries (Table 1) are generally very satisfactory, and particularly so, when it is kept in mind, that the figures represent the over-all yields of the microsynthetic and the chromatographic procedures. The notable exceptions here as well as in the microsynthesis are PTH-serine and PTH-threonine (1). These losses reflect the well-known tendency of these PTH-derivatives to undergo decomposition through dehydration. It has so far not been possible to find chromatographic conditions which would reduce these losses. The presentation of a correction for these PTH-derivatives is not considered warranted at present since sufficient material is not available as yet.

D. *Application of the procedure to a hydrolysate of bovine serum albumin*

The solution of bovine serum albumin was diluted with water to a concentration of 247.5 μg protein nitrogen per ml. Of this solution 100 μl and 200 μl samples were drawn off corresponding to 0.154 mg and 0.308 mg of bovine serum albumin if the figure 16.07 per cent nitrogen for the protein is used (9). The samples were transferred to the quartz tubes used in the microsynthesis and evaporated to dryness *in vacuo* over P_2O_5. To each sample 0.2 ml twice glass distilled hydrochloric acid (6 N) was added. The tubes were filled with nitrogen gas, sealed off, and kept at 110°C for 22 hours. After cooling the tubes were opened and the hydrochloric acid evaporated

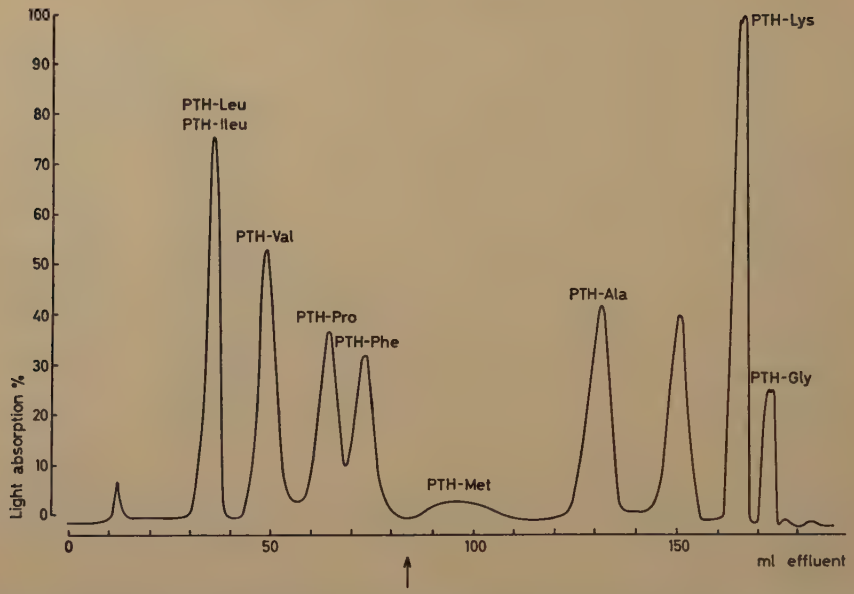

Fig. 4. Chromatographic separation of the PTH-derivatives of the amino acids from an acid hydrolysate of bovine serum albumin (0.154 mg). The arrow indicates change of solvent. Column I.

Table 2. Amino acid composition of bovine serum albumin.

Amino acid	Amino acid/100 g protein			
	This work		Stein and Moore (9)	
	grams	mmoles	grams	mmoles
Leucines	14.75[a]	112.4	14.88	113.4
Valine	6.05	51.6	5.92	50.5
Proline	4.70	40.8	4.75	41.3
Phenylalanine	6.59	39.9	6.59	39.9
Methionine	0.68	4.6	0.81	5.4
Alanine	6.34	71.2	6.25	70.1
Lysine	12.31	84.2	12.82	87.7
Glycine	1.98	26.4	1.82	24.2
Tyrosine	—	150.0[b]	5.06	27.9
Glutamic acid	—		16.50	112.2
Threonine	4.11[c]	34.5	5.83[d]	49.0
Aspartic acid	10.50	78.9	10.91	82.0
Serine	1.42[c]	13.5	4.23[d]	40.2
Arginine	6.20	35.6	5.90	33.9
Histidine	4.15	26.7	4.00	25.8

[a] Calculated as leucine.
[b] Calculated as glutamic acid.
[c] Uncorrected for losses.
[d] Corrected for losses.

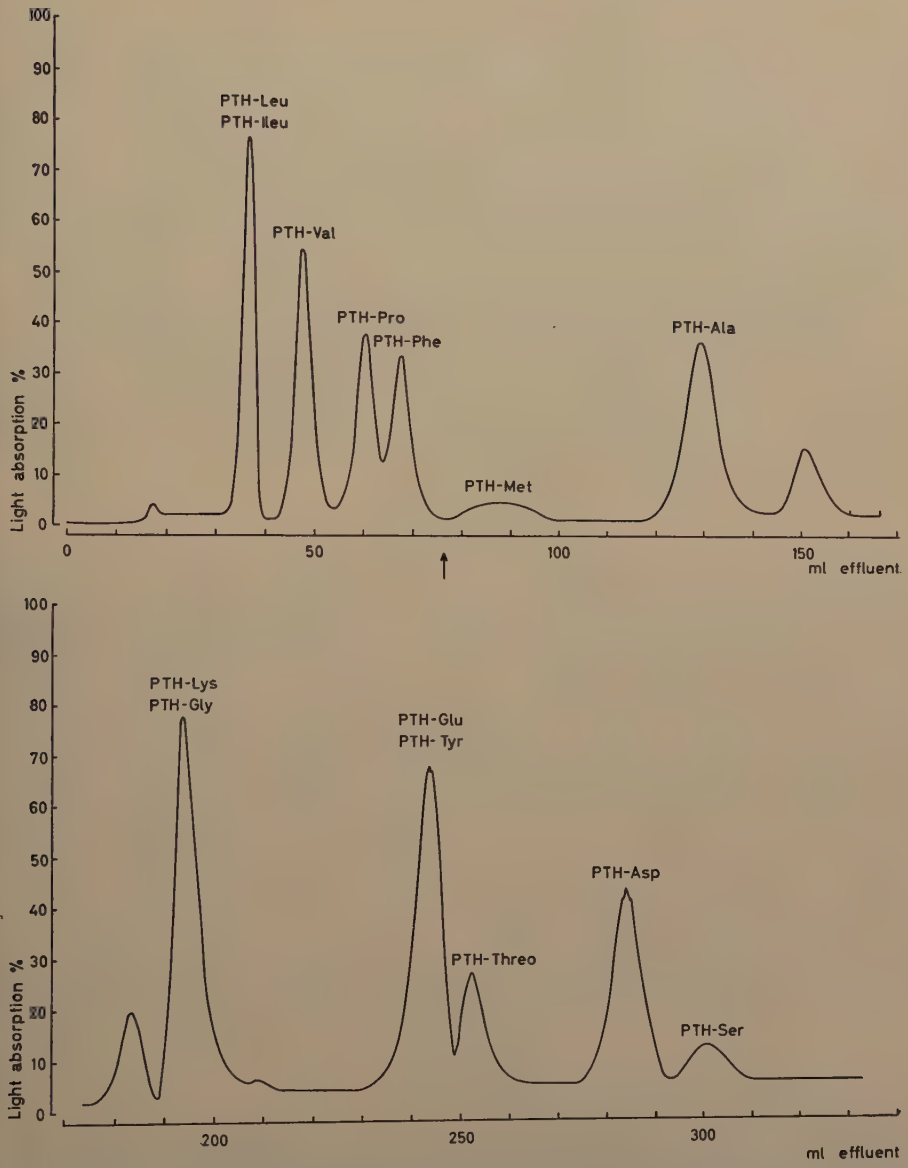

Fig. 5. Column II. Chromatographic separation of the PTH-derivatives of the amino acids from an acid hydrolysate of bovine serum albumin (0.154 mg). The gradient is set in at the arrow.

in a desiccator *in vacuo* over KOH. Water was added to each sample and this again dried in the same way. This procedure was repeated twice. The micro synthesis of the PTH's was carried out according to the directions given earlier (1).

The chromatographic procedure was the one described above.

The results of Column I and Column II are presented in Figs. 4 and 5. The analytical

results are summarized in Table 2. With regard to the appearance of the chromatograms these differ in certain respects from the appearance of the chromatograms of pure amino acids. Thus two additional peaks appear between PTH-alanine and PTH-lysine in Column II. These peaks have not yet been identified with certainty. However, the first peak, when subjected to paper chromatography (4), travels with the same R_F-value as phenyl thiourea. This peak is therefore tentatively ascribed to the ammonia present in the hydrolysate.

Furthermore, the separation of PTH-tyrosine and PTH-glutamic acid has not succeeded in this case.

In view of the low content of methionine it was necessary to use a larger sample of protein (0.308 mg) for the determination of this amino acid.

In Table 2 comparison has been made between our results of the amino acid composition of bovine serum albumin with the values given by Stein and Moore (9) who employed chromatography on starch of the free amino acids of the protein hydrolysate. In most instances the figures come fairly close to each other. However, it is too early to evaluate the merits and the deficiencies of our analytical procedure when applied to complicated amino acid mixtures.

SUMMARY

An analytical procedure for the quantitative analysis of amino acids based on the chromatographic separation of their 3-phenyl-2-thiohydantoin derivatives is presented. The course of the separation is automatically recorded by registration of the U.V. absorption in the effluent. The procedure permits the quantitative determination of glycine, alanine, valine, methionine, proline, hydroxyproline, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid, histidine, lysine, and arginine. The leucines are analyzed together. Serine and threonine give too low figures owing to decomposition of the derivatives. The material requirements for each amino acid is about 10 μg and the recoveries are practically quantitative.

The application of this procedure to a hydrolysate of bovine serum albumin is presented.

I wish to thank Dr. P. Edman for his valuable suggestions and encouragement during the course of this work. The technical assistance of my wife, Mrs. Inga Sjöquist, and of Miss Anita Welin is gratefully acknowledged.

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Temperature distribution within cylindrical electrophoresis columns of large capacity, stabilized by capillary systems

Construction of a flexible apparatus suitable for fractionations on a ten gram scale

By Jerker Porath

With 16 figures in the text

The limitation imposed by heat development and temperature distribution on the capacity of preparative electrophoresis columns and the effect of these factors on the electrophoretic resolution will be treated in the present paper. Extremely complicated relations are involved, but in the present stage of development of column electrophoresis the strict derivation of these relations is not necessary. Instead approximative data of practical use are required. The studies described are chiefly based on experiments designed to reveal the nature of temperature gradients developing under given conditions. Since only sporadic reports about temperature distribution in electrophoresis columns are to be found in the literature [1, 2] important information can be obtained by simple measurements even though the precision is not perfect.

It should also be pointed out that rough estimation of the maximum temperature within a column is of great value when proteins and other labile substances are fractionated.

As an illustration two fractionation experiments with artificial mixtures at a high current density will also be described and discussed.

Theoretical

It is essential for successful electrophoretic separation that the continuously generated heat be effectively transported from the separation region. Heat is transmitted through the medium of the column chiefly by conduction and, presumably, also by convection. Transmission through the supporting walls is effected by conduction. Radiation can be disregarded.

Heat conduction

Let us consider some expressions for the theoretical temperature distribution, which would be found if heat were transported by conduction alone. At any point of the system the heat flow at time t is determined by the equation

$$\frac{\partial T}{\partial t} = \text{div}\, \frac{\lambda}{c \cdot \varrho}\, \text{grad}\, T + \frac{Q}{c \cdot \varrho}, \tag{1}$$

where T denotes the temperature, λ the thermal conductivity coefficient, c the specific heat, ϱ the density and Q the heat generated per unit time and volume in this point. It is informative to start with this general equation for the discussion of the approximations which have to be made in order to obtain equations which can be solved.

With reasonable heat production and loading the media of the column may be regarded as isotropic from the thermal point of view and consequently c, ϱ and λ may be treated as constants. This is a reasonable assumption for the column size and the finely divided supporting solids used in zone electrophoresis. Equation (1) simplifies to

$$c \cdot \varrho \frac{\partial T}{\partial t} = \lambda \triangle T + Q, \tag{2}$$

where $\triangle$ is the Laplacean operator.

From this equation all temperature gradients can be calculated in principle. It should be possible, for example, to find the rise and decline of the gradients immediately after starting and terminating the electrophoresis, as well as axial temperature gradients. For all these cases, however, the corresponding equations are not easily integrated because, in addition to the mathematical difficulties, the boundary conditions are badly physically defined.

Even assuming certain further approximations it is only possible to easily calculate the radial temperature distribution existing at steady state. Fortunately, this distribution happens to be the most important one for the question of production capacities in zone electrophoresis columns. Thus we obtain the following relationship:

$$\frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial T}{\partial r} \right) + \frac{Q}{\lambda} = 0, \tag{3}$$

where r is the radial coordinate.

In the columns constructed by the author the cooling has been provided for in two ways. In the column type most extensively used cooling is effected by immersing the column into a cooling bath or by surrounding the column by a jacket with running water of constant temperature. In columns designed for large scale production the separation is carried out in a wide cylinder shell with inside and outside cooling. Fig. 1 shows a schematical drawing of the latter type.

The temperature at the center will be higher than close to the walls, thus the heat production Q will be dependent on the radial distance from the axis.

The variation in Q is not large, however, so we will assume it to be constant throughout the column. Equation (3) can now be integrated as an ordinary differential equation giving:

$$\frac{dT}{dr} = -\frac{1}{2\lambda} Q \cdot r + \frac{C_1}{r}, \tag{4}$$

and by a second integration we obtain the temperature T at the distance r from the axis:

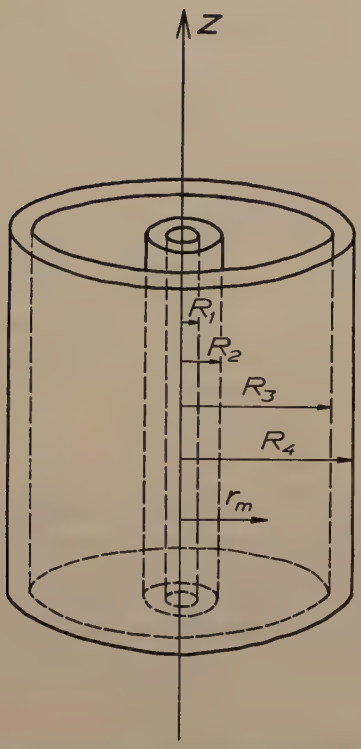

Fig. 1. Sketch of a section of an internally and externally cooled column, showing the radial distances used in the calculation of temperature distribution.

$$T_r = -\frac{Q}{4\lambda} r^2 + C_1 \ln r + C_2 \qquad (R_2 \leq r \leq R_3). \tag{5}$$

The determination of the integration constants C_1 and C_2 involves no difficulties if we make use of the fact that all heat produced inside the shell bounded by the cylinder surfaces $r = r_m$ (r_m being the radial distance from the axis to the point where the temperature reaches maximum) and $r = R_2$ must leave the column through the inner tube and the heat produced outside $r = r_m$ is conducted through the outer tube. We assume the apparatus to be constructed of tubes of the same material with the thermal conductivity coefficient λ_g and with the inner and outer radii R_1, R_2, R_3 and R_4 as indicated in Fig. 1 and that the temperatures at the corresponding surfaces are T_1, T_2, T_3 and T_4. If the thermal conductivity coefficient of the column content is λ_c we obtain the following relations:

$$r_m = \sqrt{\frac{\lambda_c}{Q \cdot A} (T_4 - T_1) + B/A} \tag{6}$$

and

$$T_r = T_4 + \frac{Q}{2\lambda_g} (R_3^2 - r_m^2) \ln R_4/R_3 + \frac{Q}{4\lambda_c} (R_3^2 - r^2) + \frac{Q r_m^2}{2\lambda_c} \ln r/R_3, \tag{7}$$

where $$A = \tfrac{1}{2} \ln R_3/R_2 + \frac{\lambda_c}{2\lambda_g} \ln \frac{R_4}{R_3} \cdot \frac{R_2}{R_1}$$

and $$B = \tfrac{1}{4} (R_3^2 - R_2^2) + \frac{\lambda_c}{2\lambda_g} (R_3^2 \ln R_4/R_3 + R_2^2 \ln R_2/R_1).$$

If the column were filled with electrolyte solution alone the heat generated expressed in calories per cm^3 per sec would be determined by

$$Q = 0.239\ i^2/\varkappa, \tag{8}$$

where i is the current density (amp cm^{-2}) and $\varkappa$ is the conductivity of the electrolyte solution (ohm^{-1} cm^{-1}). The effective cross sectional area is reduced by the stabilizing uncharged substance so that the heat generated becomes

$$Q = k \cdot 0.239\ I^2/q^2\varkappa, \tag{9}$$

where k is a proportionality factor characteristic for the packing material, I the current (amp), and q the cross sectional area of the column (cm^2).

For the simple but important case where the cylindrical column is cooled only by a surrounding water jacket with circulating water of constant temperature, equation (7) reduces to a very simple expression. If T_m is the maximum temperature and T_r the temperature at the distance r from the axis, we get

$$T_m - T_r = \frac{Q \cdot r^2}{4\lambda_c}. \tag{10}$$

If Q now is substituted in this equation, the following relation is obtained:

$$T_m - T_r = KI^2r^2/R^4\varkappa, \tag{11}$$

where $$K = k \cdot 0.239/4\pi^2\lambda_c \tag{12}$$

and R is the inner radius of the column tube.

The difference between the temperature at the periphery T_0 and at the center, T_m is

$$T_m - T_0 = KI^2/\varkappa R^2. \tag{11a}$$

It will be shown that K is approximately 4.6 in cellulose columns. The equation is very useful for rough estimation of permissible current values.

An equation similar to (11) was used by Peniston *et al.* for calculation of the temperature rise within agar gel columns [2].

Convectional heat transport

Density gradients in a liquid cause gravitational instability and give rise to turbulent currents. Density variations between different points of a liquid depend partly on uneven distribution of material and partly on temperature gradients. In electrophoresis columns the first factor naturally becomes important when there is extensive loading or in the presence of salt gradients, while temperature gradients are formed

at high current density. Material transport by turbulence is naturally followed by heat transport. Of interest here is the extent to which an effectively stabilizing capillary system is able to reduce convection. An idea of the importance of convection may be obtained by comparing the temperature distribution found experimentally with that calculated from the heat conduction equation. If convection is of importance the difference between these values should increase with increased heat generation, viz. current strength. This will be exemplified by experiments in glass bead columns.

If the entire liquid moves relative to the stabilizing medium forced convection also occurs. This factor depends on the flow of liquid, density gradients and the structure of the capillary system. An understanding of the influence of forced convection may be obtained empirically.

Experimental

A. *Apparatus*

The apparatus has been developed from a similar type described earlier [3], but deviates from the latter in so many respects that a thorough description of its details is required. Assembly and operation are simpler and are performed with less risk of damage to breakable parts. Largely this has been made possible by the avoidance of large ground glass joints. Connections of a new type have been introduced. This has been made possible by an interesting observation by Mr. N. Larsson of this Institute that hot perspex and pyrex glass tubes[1] of proper dimensions may be permanently joined to form strong joints. These joints resist not only very large mechanical strains, but also prolonged drying alternating with wetting with aqueous solutions. The new principle of construction has been found to be very suitable for column diameters of 5–8 cm, but may probably also be used for still larger columns. The apparatus described in detail here has an inner column diameter of 6.8 cm. It has been used for electrophoretic experiments, while the column used for temperature measurements has an inner diameter of 8.0 cm.

The column (*A*) (*Fig. 2*)

Electrophoresis is performed in the pyrex tube (*A*1) (length 105 cm; inner radius 3.4 cm; wall thickness 0.4 cm). The content of the column is supported by a nylon net (*A*2), on which rests a thick filter paper disc.

The net is held in place by a short perspex collar (*A*3) which is joined directly to (*A*1). In preparing the net, the nylon fiber is allowed to swell in water at room temperature for a few days. The wet fiber is then wounded as tightly as possible. The winding and fixation of the net to the perspex collar are shown in Figs. 2 and 3. (*A*3) is threaded so that the end piece (*G*) or the short extension column (*B*) may be connected (Fig. 3). The surface of (*A*3) is provided with grooves for a wrench (indicated by unfilled squares in the figures).

The electrophoresis tube (*A*1) is surrounded by a water jacket, the outer wall of which (*A*4) consists of a perspex tube (radius 5.0 cm; thickness 0.90 cm) with the lower

[1] The tubes of perspex and glass are simultaneously warmed to ca. 100°C in oven. The soft perspex tube is pressed onto the glass tube and is fixed to the latter by the contraction on cooling.

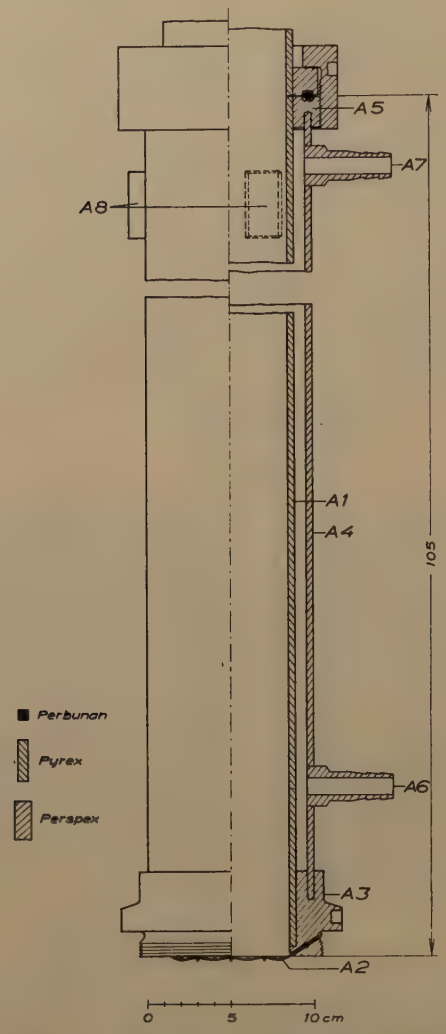

Fig. 2. The column tube (*A*1) with cooling jacket (*A*4). The column content is supported by the nylon net (*A*2). The lower end of the column can be immersed into electrolyte solution or connected to the end piece (*G*) or the extension column (Fig. 3). The upper end is connected to (*C*) or (*D*) (Figs. 4 and 5) at (*A*5).

end pressed into a track and cemented to (*A*3). Its upper end is connected in a similar way to a perspex collar (*A*5). (*A*4) is provided with an inlet (*A*6) and outlet (*A*7) for cooling liquid, as well as perspex projections cemented onto the tube at 120° angles apart for support in hanging the column.

The upper part of the column is coupled to the connecting piece (*C*) (Fig. 4) or the "head" (*D*) (Fig. 5) by means of a sleeve joint (*C*3) or (*D*3), which may be screwed over (*A*5). (*C*2) or (*D*2) are thus pressed onto (*A*5), allowing the connection to be

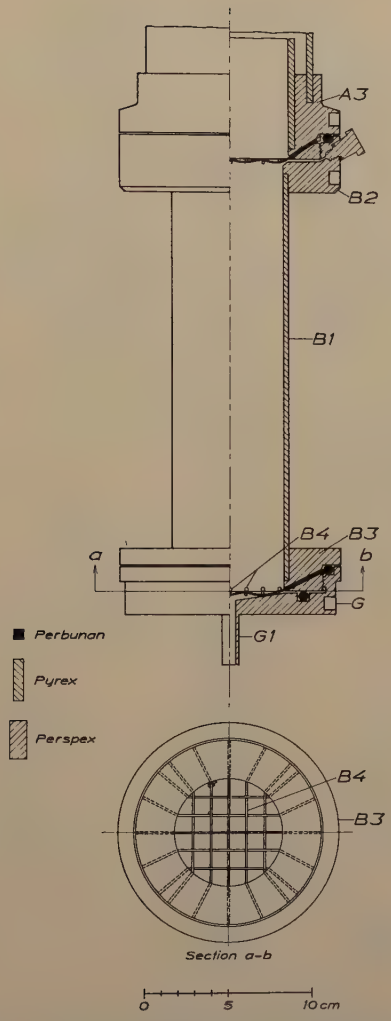

Fig. 3. Extension column (*B*) with end piece (*G*). The extension column is connected to (*A*) (Fig. 1) when upward migration of the substances is desired, e.g. when countercurrent flow of liquid is used for compensation of the electrophoretic migration.

fully sealed by means of a ring-formed perbunan gasket (Simrit 'O'-ring; inner radius 4.5 cm; thickness 0.5 cm)[1] fixed in a track at the surface of contact.

Short extension column (*B*) (*Fig. 3*)

The electrophoresis tube (*A*1) can be extended by means of a tube (*B*1) (length 28.5 cm, radius 3.0 cm, thickness 0.35 cm). Coupling is made possible by the perspex collar (*B*2) with inner threads fitting (*A*3). Sealing is obtained by a perbunan ring

[1] Perbunan is an oil-resistant synthetic rubber. The perbunan parts here referred to have been obtained from Carl Freundenberg, Simrit-Werk, Weinheim/Bergstrasse, Germany.

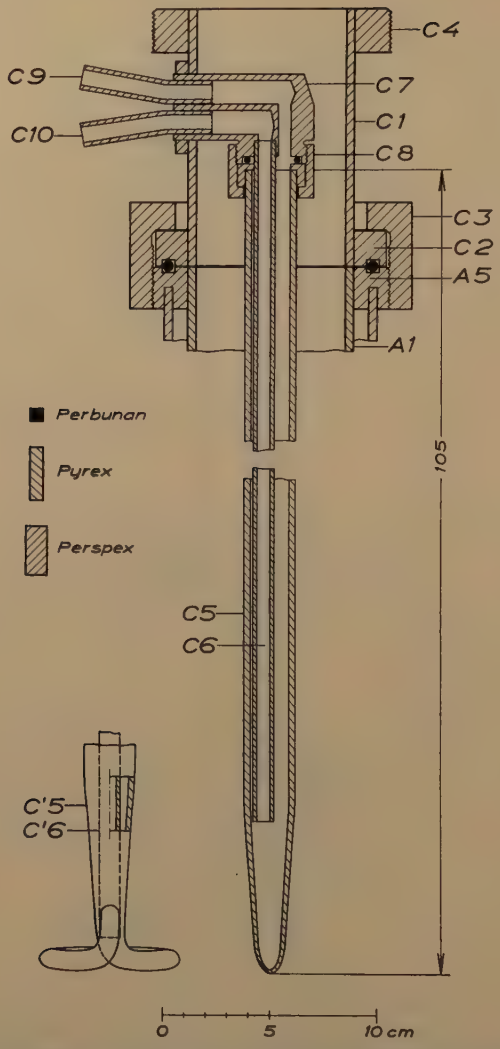

Fig. 4. Connection piece. This part is inserted in between the column (*A*) (Fig. 2) and the head (*D*) (Fig. 5) when central cooling is desired. The cooling tube is a straight tube ending in a tip (*C*5) or a loop (*C'*5). The cooling tube is connected to the tube wall (*C*1) via the bridge (*C*7).

gasket (Simrit 'O'-ring, inner diam. 5.5 cm; thickness 0.5 cm). In screwing these pieces together, the excess buffer passes out through a hole in (B2), which is then closed with a screw of perspex or nylon. At the end of (*B*1) there is a threaded perspex collar (*B*3) with a nylon net (*B*4).

The connection piece (*C*) (*Fig. 4*)

A connecting piece is used when central cooling is desired. It consists of a 8 cm long perspex tube (*C*1) of the same diameter and thickness as (*A*1) and is provided

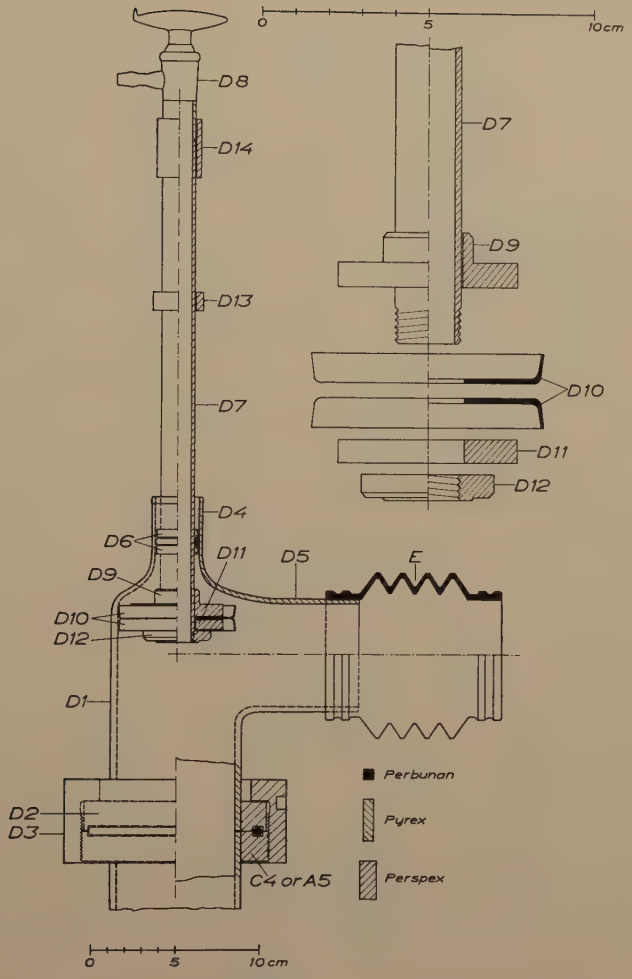

Fig. 5. The head of the column which connects (*A*) or (*C*) (Figs. 2 and 4) with a corrugated perbunan tube (*E*) which in turn is connected to (*F*) (Fig. 6). The piston tube (*D*7) with the gaskets (*D*10) and supports (*D*9), (*D*11) and (*D*12) makes possible liquid connection and disconnection between the two limbs of the apparatus. When closing the connection the piston is pushed downward so that (*D*10) seals (*D*1).

with threaded perspex collars (*C*2) and (*C*4). A sleeve joint (*C*3) is screwed onto the threads of (*A*5) over (*C*2). The glass central cooling tube (*C*5) (length 105 cm; outer radius 1.05 cm, thickness 0.2 cm) is connected to the inner tube (*C*6) at (*C*7) with a sleeve joint (*C*8), as indicated in the figure. The bridge (*C*7) is a right-angled perspex tube containing two channels and is fastened to the tube (*C*1). The channels are connected to the inlet and the outlet for cooling water (*C*9) and (*C*10).

The bridge (*C*7) and the tube (*C*1) were first constructed of glass but cracks often formed at the bend. Hence the construction described is preferable in spite of the less effective heat conductance of perspex.

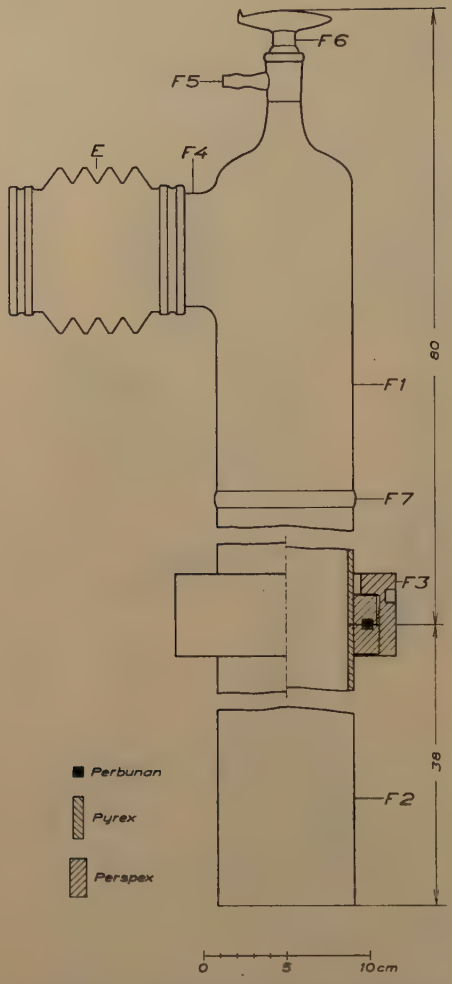

Fig. 6. The connecting arm which is attached to (*D*) (Fig. 5) via (*E*). The lower part of (*F*2) is immersed into an electrode vessel. The length of (*F*) may be varied with interchangeable tubes (*F*2) of different length, for matching columns of different length or for permitting application of large hydrostatic pressure differences.

The cooling tube (*C*5) and (*C*6) may be bent in a loop at its lower end. As the loop will be in contact with or close to the nylon net, an efficient cooling of the bottom of the column is thus provided for.

Top piece (*D*) (*Fig. 5*)

A short tube (*D*1) of the same diameter and thickness as (*A*1) is provided at one end with a perspex collar (*D*2), and a sleeve joint (*D*3). The other end is drawn out to a narrower open tube (*D*4) (length 2.4 cm; inner radius 1.12 cm; thickness 0.26 cm).

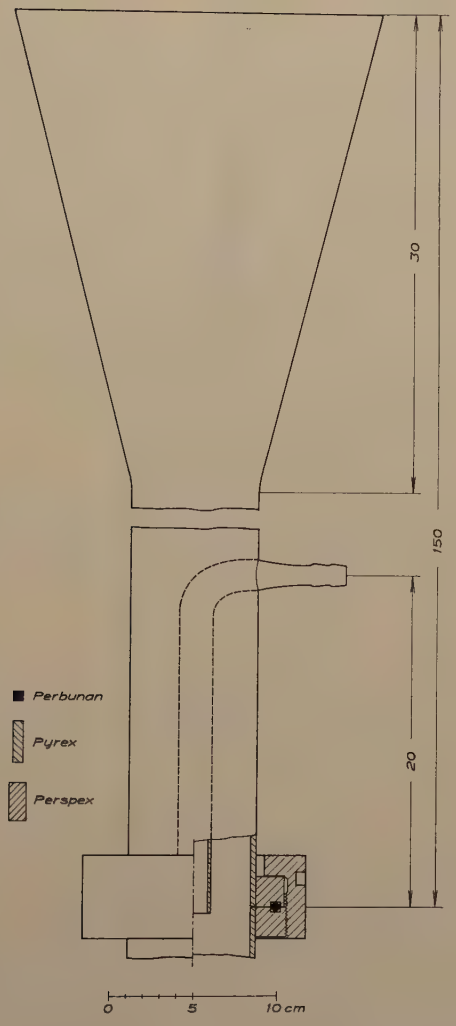

Fig. 7. Packing funnel. When packing, the funnel is placed on top of the column tube (*A*) or the connection piece (*C*).

Perpendicular to (*D*1) is a side tube (*D*5) (length 5.5 cm; inner radius 3.2 cm; thickness 0.2 cm).

The lower planed surface of the perspex collar (*D*2) is provided with a circular groove for a gasket. Two glass rings (*D*6) are united with the tube (*D*4) and are each 0.5 cm wide and 0.15 cm thick and 0.5 cm apart. A gasket (Simrit 'O'-ring; inner radius 0.9 cm; thickness 0.25 cm) is inserted between the glass rings (*D*6). This gasket seals the piston (*D*7)—a perspex tube (length 27 cm; inner radius 0.75 cm; thickness 0.25 cm) the upper end of which is provided with a stopcock (*D*8). The outside of the lower end of (*D*7) is threaded for cementing or screwing on a disc (*D*9). Two similar

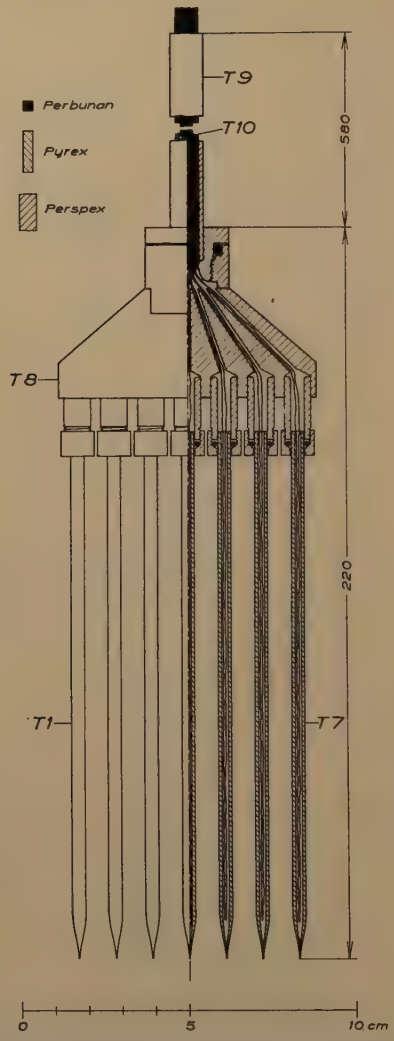

Fig. 8. Device for temperature measurements. During temperature measurements the piston (D) with accessories is exchanged for this fork. ($T9$) slides smoothly against the 'O'-ring at ($D6$) (Fig. 5) thus making movement in the axial direction possible. The thermocouple joints are distributed to different radial positions in the powder column by means of ($T8$) and the tubes ($T1$)–($T7$).

gaskets ($D10$) (Simrit T70-16; central hole enlarged in order to fit the piston) are placed between ($D9$) and a flat, wide perspex ring ($D11$). Then ($D10$) and ($D11$) are fixed by the screw ($D12$).

When the piston is pushed down so that the collar ($D13$) at ($D7$) rests against ($D4$), ($D10$) seals the lower part of ($D1$). One end of a 3.5 cm long perspex coupling tube ($D14$) is connected to a short glass tube provided with a stopcock ($D8$). The tube ($D7$) is cemented to the other end of ($D14$).

The connecting arm (*F*) (*Fig. 6*)

This consists of 2 tubes; (*F*1) (pyrex glass; length 80 cm; inner radius 4.0 cm; thickness 0.3 cm) and perspex (*F*2). For making hydrostatic compensation [3] possible over a wide range, two or more tubes (*F*2) of different length may be interchanged. (*F*1) and (*F*2) are connected by a sleeve joint (*F*3) constructed as described earlier.

(*F*) is connected to (*D*) by means of (*E*), a corrugated perbunan tube (Simrit LM-21415, type V6-242; Simrit-Werk catalogue no. 660). One end of this is pushed onto (*D*5) and the other on the arm (*F*4) (length 6.5 cm; inner radius 3.2 cm; thickness 0.3 cm). (*F*1) is reduced at its upper end and there provided with a ground glass joint (standard no. 19) with a side tube (*F*5). (*F*) may be opened to the air or closed by turning the stopcock (*F*6), which has an opening in the joint toward (*F*5).

The perspex ring (*F*7), cemented to (*F*1) serves as a support for hanging up the apparatus.

The end piece (*G*) (*Fig. 3*)

When the column is not in use, the lower end of (*A*) or (*B*) is closed by the end piece (*G*). The tube (*G*1) is provided with a tube and a clamp. (*G*) is also used when continuous registration of the conductivity, optical density, etc., of the effluent is desired.

Packing funnel (*Fig. 7*)

The lower end of a funnel suitable for use in packing the column is connected to (*A*5) or (*C*4). After the packing has terminated, the remaining suspension is emptied through the side tube.

Thermometer fork (*Fig. 8*)

Elements of the thermocouples may be inserted into the column in a fork-like sheath. Seven thin-walled glass tubes (*T*1–*T*7) (length 16 cm; inner radius 0.19 cm; thickness 0.03 cm) are fastened on to a flat "distributor" of perspex (*T*8). Each tube is connected to the shaft (*T*9) through a channel. (*T*9) is so constructed as to slide against the ring at (*D*6) so that (*T*8) can be pushed down through (*D*) toward the surface of the supporting material.

Additional equipment

Platinum electrodes and electrode vessels are the same as described earlier [3]. Electrolyte solution in the electrode vessels are continuously mixed by circulation. A rectifier capable of yielding a current of 1 A and a voltage of 3500 V is used as a power supply.

B. *Assembly and packing of the column. Electrophoresis exemplified by fractionation of an artificial glycine–glutamic acid mixture*

We shall here attempt to give a rather complete description of the assembly and packing of the column as well as the application of the material to be fractionated and its subsequent electrophoresis and displacement from the column. For additional details the reader is referred to earlier publications from this Institute [3, 4].

Assembly and packing

A steel support (preferably secured to both floor and ceiling) is provided with a strong clamp and holder. The former consists of a wide ring with two halves to one of which is attached a shaft. The two ring halves are connected using the wing screws so that the column is surrounded by the ring and the latter rests on the perspex supports, (*A*8). A thick filter paper disc of the same diameter as the tube (*A*1) was introduced into the latter and pushed down until it came to rest on the net, thereby sealing the lower opening of the tube. (*G*) was screwed onto (*A*3) and a long rod with a wide cork on one end was inserted into the tube until it came to rest on the filter paper disc. The column was filled with evacuated buffer solution by siphoning through (*G*1) until the buffer level rose to within a few cm from the upper end of (*A*1). (*C*) was screwed on after all threads had been well lubricated with grease. Lubrication of the threads of the more permanent connections must be performed with care in order to prevent them from sticking together. The funnel for packing was connected at (*C*4). The buffer level was raised until the funnel tube was completely filled. A mechanical stirrer was mounted over the funnel after which the latter was carefully filled with a thin, evacuated suspension of Munktell paper powder[1] in 0.1 molar pyridinium acetate solution [3]. The suspension was strongly stirred and the powder allowed to sediment down through the tube. A clamp on a rubber tube attached to (*G*1) prevented vertical displacement of the solution. When a layer of sediment 3–4 cm in height had formed, (*G*) was removed and the sedimentation was allowed to proceed with a simultaneous vertical flow of solution through the column as recommended by Flodin and Kupke [4]. The packing funnel was filled several times with additional paper powder suspension. The suspension was made very thin in order to eliminate the disturbing influence of the bridge (*C*7). When the layer of sediment had reached the desired height (1–2 cm from the upper end of (*A*1)) the length of the cellulose column was 104 cm. The excess suspension was removed by opening the side tube of the packing funnel after which the tube was once more closed. The packing funnel was now filled with buffer solution and the column was washed with 4–5 liters. The funnel was subsequently replaced by the top section (*D*) and (*D*10) and the lower end of (*D*7) were lubricated. With the stopcock, (*D*8), open the piston was pushed down until (*D*10) contacted (*D*1) after which the stopcock was closed.

A tube (*F*2) was coupled to (*F*1) and a PVC tube (diameter 1.0 cm) was introduced from below through (*F*2) and (*F*1), out through (*F*4) and once more in through (*F*4) down through (*F*1) and (*F*2). Cooling water was circulated through the tube. (*F*) was hung up on the same stand as (*A*) with (*F*3) and (*E*7) resting on a support of the same type as described in connection with (*A*). (*F*) is joined to (*D*) using the perbunan tube (*E*). The loop of the cooling tube then went through (*E*), with the bend in (*D*5). The ends (*D*5) and (*F*4) were lubricated in order to facilitate connection. An electrode vessel was introduced under (*F*) and filled with buffer. The buffer was raised by connecting (*F*5) to a water suction pump. After filling (*F*), (*F*5) was closed (see Fig. 9). Each electrode vessel was filled with 7 liters of 0.1 molar pyridinium acetate solution.

The application and electrophoretic separation of the glycine–glutamic acid mixture

The amino acid mixture.—4 g of glutamic acid and 20 g of glycine plus 10 mg of DNP-aspartic acid[2] were dissolved in 100 ml water. The conductivity of the aqueous

[1] Available from Grycksbo Pappersbruk, Grycksbo, Sweden.
[2] DNP- is an abbreviation for 2,4-dinitrophenyl-.

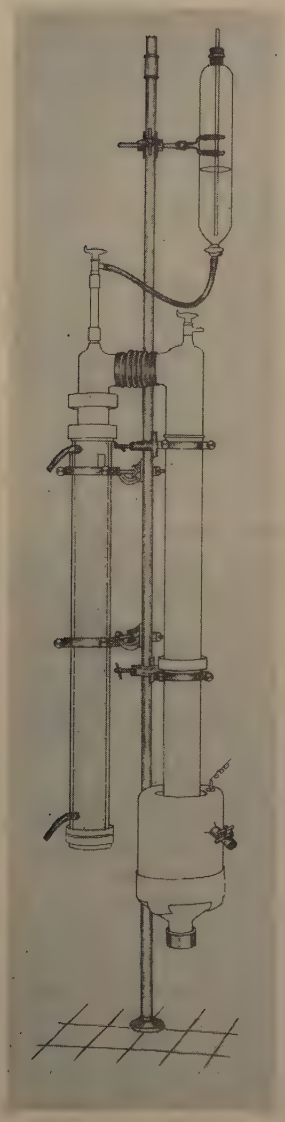

Fig. 9. Apparatus assembly. The electrode vessel under (*A*) is removed. The cooling tube in (*F*) and the plug (*D*9)–(*D*12) are omitted for simplicity. See also description in the text.

solution was 1.7×10^{-3} ohm^{-1} cm^{-1} or lower than that of the pyridinium acetate solution (which was 2.4×10^{-3} ohm^{-1} cm^{-1} at the same temperature).

(*G*) with attached rubber tube and clamp was screwed to (*A*3). The stopcock (*D*8) was removed from the ground glass joint and the buffer solution between (*D*10) and the upper surface of the cellulose was removed. The amino acid solution was introduced through (*D*7) with a syringe to which a plastic tube had been attached and was allowed to flow into the column slowly, the flow being regulated by means of the clamp under (*G*). The amino acid solution was washed into the column with

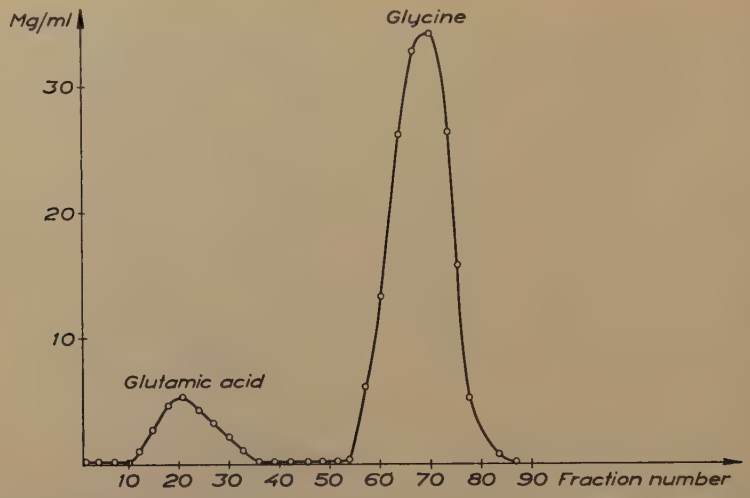

Fig. 10. Electropherogram showing the separation of a mixture of 4 g glutamic acid and 20 g glycine in 0.1 molar pyridinium acetate. Starting zone 100 ml. Electrophoresis was performed for 29 hours at a current of 500 mA, corresponding to a temperature difference of 8.5°C between the center and the wall. Each fraction contained 30 ml.

buffer until the lower border of the yellow zone lay 12 cm from the upper surface of the cellulose. The approximate width of the zone was 5 cm.

The buffer was filled up to (*D*8) and the stopcock once again placed in position. (*G*) was removed from (*A*3). The apparatus at this stage is shown in Fig. 9. An electrode vessel was placed under (*A*) and filled with buffer solution. The electrode (*A*) was connected to the positive pole and the electrode under (*F*) to the negative pole of a rectifier. The electrode vessels were connected by means of a siphon and also through a diaphragm pump.

(*D*10) was carefully lifted up into the position shown in Fig. 5. A voltage of 1050 V was applied to the electrodes resulting in a current of 520 mA. The buffer was circulated between the electrode vessels at a rate of 130 ml/min. During the first phase of the electrophoresis spreading of the yellow zone occurred, the zone then became narrower, and finally a slow spreading once more was observed. After 29 hours the yellow zone was 15 cm distant from the net. The pump was stopped and the siphon removed. The current through the column was then found to be 500 mA. The lower end of the column was quite warm. The electrophoresis was discontinued.

Samples of buffer from the two electrode vessels were taken. Analysis yielded the following:

Anolyte: $\varkappa$ 2.40 × 10^{-3} ohm^{-1} cm^{-1}, pH 4.9; catholyte: $\varkappa$ 2.43 × 10^{-3} ohm^{-1} cm^{-1} pH 4.9.

(*D*8) was connected to a buffer reservoir. The connection between the two limbs of the apparatus was disconnected by lowering (*D*10) into (*D*1). The connection to the buffer reservoir was disconnected. The electrode vessel under (*A*) was emptied and moved aside. A fraction collector was placed under (*A*). The buffer reservoir was connected again, and the height of the latter adjusted so that the rate of flow of

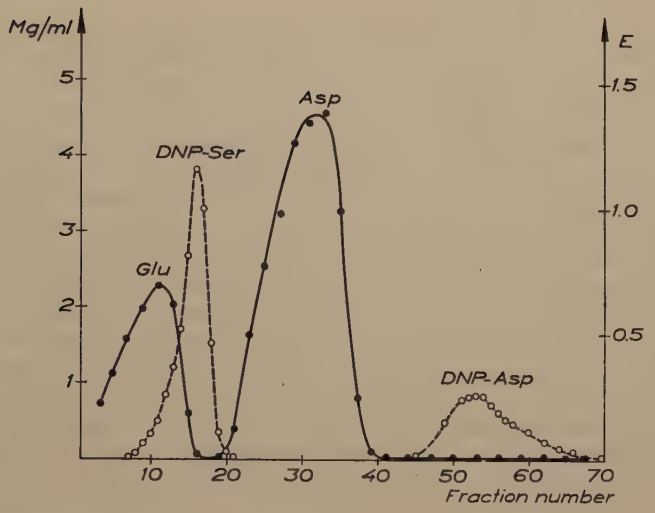

Fig. 11. Electropherogram showing the separation of 1.4 g glutamic and 3.2 g aspartic acid and two "markers" DNP-serine and DNP-aspartic acid 10 mg each. The sample (in 150 ml) was placed in the extension column (*B*) and electrophorized upwards. After 48 hours the electrophoresis was compensated by a counter current liquid flow. The total run extended over 74 hours. The maximum temperature difference within the column during the last 48 hours was approximately 8.5°C. Each fraction contained 50 ml. Further details in the text.

solution from the column was 180 ml/hr. Fractions of 30 ml were collected. 4 ml aliquots were taken from each fraction and were concentrated to dryness in weighed polyethylene cups. The weight curve was determined (Fig. 10). The material in the fractions containing glutamic acid and glycine was recovered in crystalline form by concentration. The yield for glycine was 20.2 g, for glutamic acid 3.97 g.

C. *Fractionation using a hydrodynamic counter flow*

The amino acid mixture.—1.5 g glutamic acid, 3.2 g aspartic acid, and 10 mg each of DNP-aspartic acid and DNP-serine were dissolved in 150 ml of water. The conductivity of the solution at 0.5°C was 0.84×10^{-3} ohm^{-1} cm^{-1}.

The connecting piece, (*C*) with cooling tube ending in a loop (Fig. 4), was connected to the column (*A*). As previously, the column was filled with 0.1 molar pyridinium acetate and was connected to (*D*), (*E*), (*F*) and the electrode vessel under (*F*). The short column tube (*B*) was packed in the same way as the large column. The amino acid solution was filtered into (*B*) followed by 25 ml of the electrolyte solution. With the channel in (*B*3) open and solution above the cellulose, (*B*3) was connected to (*A*) after which the channel was closed. The electrode vessel was introduced below (*B*) and filled with buffer solution. The buffer levels in the two electrode vessels were equalized by means of a siphon and the electrolyte solution was circulated as in the previous experiment.

The electrode in the vessel under (*B*) was connected to the negative pole, and that in the vessel under (*F*) to the positive pole of the rectifier and 650 volts potential difference was applied, resulting in a current of 200 mA. After 26 hours two yellow zones could

be seen in the lower part of the column (*A*). The lower zone (containing DNP-serine) was approximately 15 cm from the bottom of the column (*A*5). The experiment was discontinued. (*B*) was disconnected after which the electrophoresis was continued at 1040 V (520 mA) with the same buffer level in both electrode vessels. After 12 hours the zone of DNP-aspartic acid was approximately 30 cm from the top of the column. The experiment was once again discontinued and the electrode vessels were placed in such a way that the anolyte ran over into the cathode vessel when the difference in levels of the two buffer solutions exceeded 4 cm. The buffer was circulated as before and the current was applied (520 mA). By combining the effects of compensation and electrophoresis the DNP-serine was forced back at a rate of approximately 1 cm per hour. After a while the zone of DNP-serine became narrower while the upward migrating zone of DNP-aspartic acid continued to spread. 74 hours after the start the experiment was discontinued and the solution in the column displaced as in the previous experiment. 50 ml fractions were collected every 10 minutes. The DNP-amino acid zones were measured at 360 mμ. 4 ml aliquots of each fraction were concentrated to dryness and weighed. The distribution of the various amino acids is indicated in Fig. 11.

D. *Determination of temperature gradients*

The temperature measurements were carried out in a column without central cooling. The column was 65 cm long and had a radius of 4 cm. In principle the construction was similar to the apparatus shown in Fig. 9. All measurements were

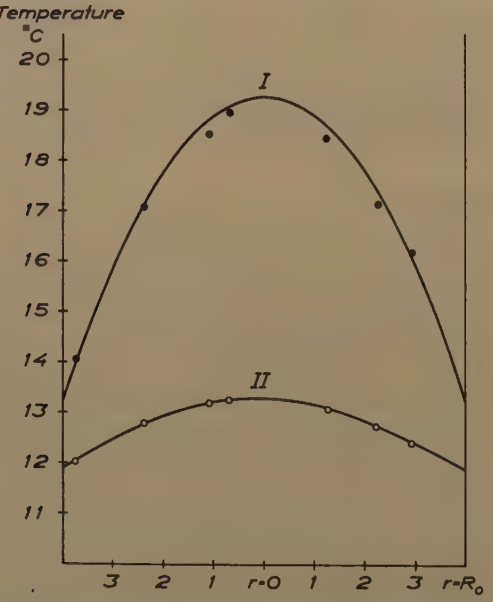

Fig. 12. Temperature distribution in an externally cooled cellulose column (diameter 8 cm) at a current of 275 mA (curve I) and 137 mA (curve II). The circles represent experimentally found temperature values at 12 cm below the cellulose surface. The lines are the theoretical curves calculated from eq. (11) ($K/\varkappa = 1280$).

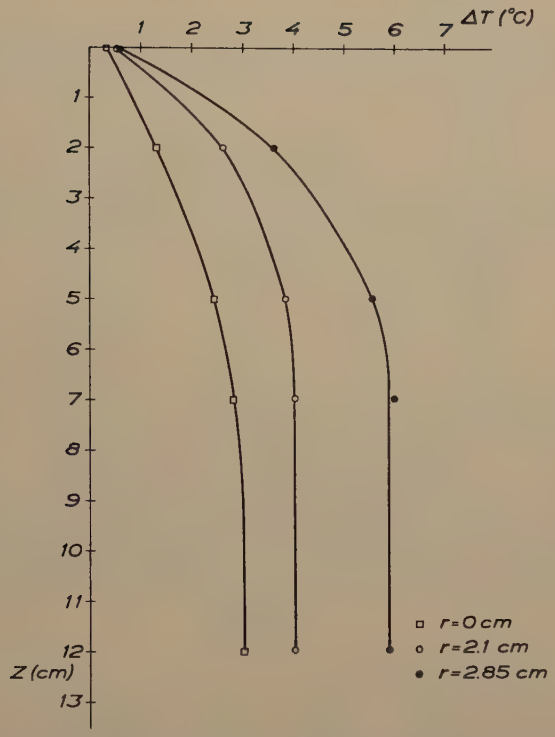

Fig. 13. Experimentally found longitudinal temperature distribution at the top of the same cellulose column as referred to in Fig. 12. The curves represent temperature gradients at different radial distances.

performed with 0.1 molar pyridinium acetate solution as the electrolyte. The following stabilizing media have been studied: cellulose powder (Munktell), potato starch (Amend[1]) and glass beads (Ballotini no. 18,[2] radius: < 10 mm).

Cellulose

The temperature at different levels of the column relative to the upper surface and at varying distances from the axis of the column was determined with thermocouples using the apparatus described above (Fig. 8). The reference arm of the thermocouples was dipped into the cooling water, the temperature of the latter being measured with a mercury thermometer. The fork was driven down to a given depth in the cellulose bed. A 290 volt potential difference was applied and the current was found to be 275 mA. The current in the thermocouple was measured with a calibrated reflecting galvanometer which could be successively coupled to the various elements of the thermocouples in the fork. The temperature distribution which had developed was measured at various intervals. When temperature equilibrium was reached the fork was driven further into the cellulose and a new series of measurements was performed, etc. The depth and radial position of the thermocouple elements could both be

[1] Amend Drug and Chemical Co., In., New York, N.Y., U.S.A.
[2] Chance Brothers Ltd. Smethwick 40, Birmingham, England.

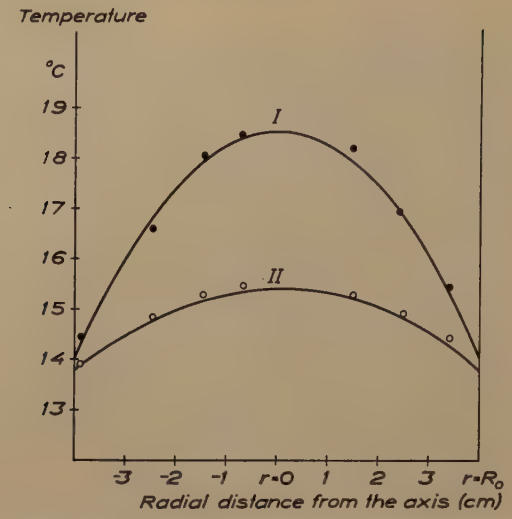

Fig. 14. Radial temperature distribution in starch column 12 cm below the surface of the starch (same dimensions of the columns as in Fig. 12). The curves I and II have been obtained from eq. (11) ($K/\varkappa = 3200$) for $I = 150$ mA and 90 mA respectively. The circles represent experimental values.

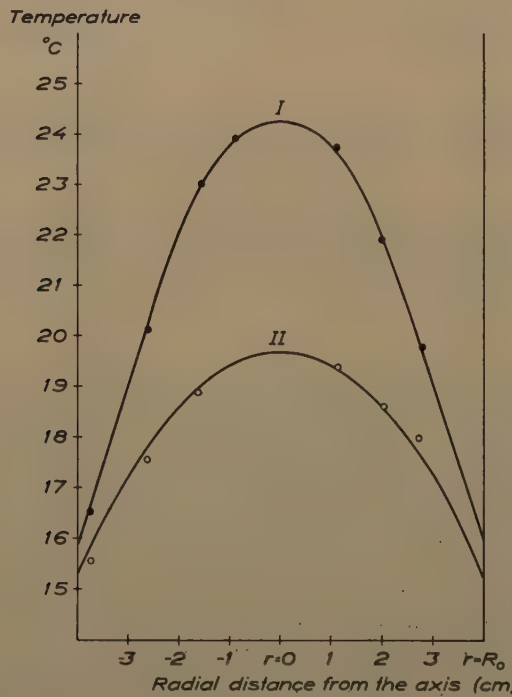

Fig. 15. Temperature distribution in glass bead columns (Ballotini No. 18) 12 cm below the surface of the stabilized column. Curve I is obtained from eq. (11) for $I = 275$ mA and $K/\varkappa = 1730$; curve II also from eq. (11) for $I = 190$ mA but $K/\varkappa = 1930$. The circles represent experimental values.

measured with a precision of ± 0.1 cm. The results of these measurements are shown in Fig. 12 (curve I) and Fig. 13. Measurements at a depth of 12 cm were also performed at 137 mA (Fig. 12 curve II).

Starch and glass beads (Ballotini)

The column tube was filled with a suspension of starch or glass beads (satisfactory packing was obtained without the use of the packing tube). Radial temperature distribution at a depth of 12 cm was measured at 2 current strengths in both cases (Figs. 14 and 15).

Determination of k (equation (9))

k at 15°C was determined for columns of 0.1 molar pyridinium acetate solution stabilized with cellulose, starch and glass beads. The apparatus was first filled with only buffer solution. A series of measurements of the current strength at different potential differences (no siphon or pump) was made. Similar series were performed with columns filled with suspensions of cellulose powder, starch or glass beads. The average value for k was calculated from the dimensions of the columns: $k_{\text{cellulose}} = 1.45$; $k_{\text{starch}} = 4.12$; $k_{\text{glass beads}} = 3.45$.

Discussion

Temperature gradients

If we assume that $K/\varkappa$ (equation (11 a)) is constant within the temperature interval for $T_m - T_0$ encountered in electrophoresis, a straight line is obtained when $T_m - T_0$ is plotted against $(I/R)^2$. Fig. 16 shows that this assumption is justified for cellulose columns under the conditions of these experiments. From the slope of the line, $K/\varkappa$ is found to be 1280 and $K = 4.6$ ($\varkappa_{15°C} = 3.6 \times 10^{-3}$ ohm^{-1} cm^{-1}).

Temperature measurements in a column of smaller diameter were performed in an earlier study [3]. The disturbing influence of the glass tube containing the thermocouples on the electric and thermal fields is an apparent source of error in all temperature measurements. This factor is presumably significant in columns of relatively small radius.

Orr and Dalla-Valle [5] observed that electric and thermal fields are similarly influenced by the proportion of solid and fluid phases. It is therefore reasonable to suppose that the ratio between the proportionality factor k and the electrical conductivity within the temperature interval involved here is constant to a first approximation. Curves I and II, Fig. 12, are graphic representations of equation (11) with $K/\varkappa = 1280$, I being 275 and 137 mA, respectively.

With the current strengths employed here the experimentally derived temperature values at a depth of 12 cm in the column and at various distances from the axis fall within the margin of error of the measurements on the curves calculated from equation (11).

In cellulose columns of dimensions used up to now the temperature distribution may at least formally be described exactly as a function of heat transport through heat conduction.

Equation (11) with the same $K/\varkappa$ probably holds also for columns with radii greater than 4 cm (see Fig. 15). It should therefore be possible to further increase the capacity

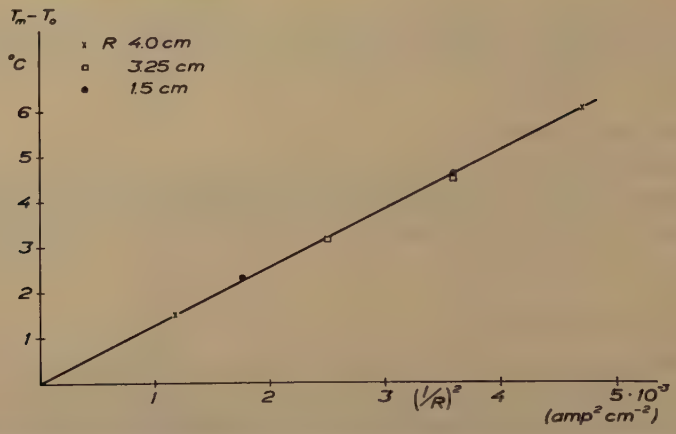

Fig. 16. The temperature difference between center and wall in various cellulose columns plotted as a function of $(I/R)^2$. The line is the theoretical curve obtained from eq. (11a) with $K/\varkappa = 1280$. The experimental values are marked as circles, squares or crosses.

without central cooling. The prolongation of the electrophoresis time necessary in such a case to obtain a given separation can be avoided by more effective cooling if a central cooling tube is inserted into the cellulose bed. Larger amounts of material, for example labile proteins, may thus be separated in a shorter interval of time.

As a result of the increase in temperature of the cooling water during passage along the length of the column a weak temperature gradient is formed. If the rate of flow is adjusted so that the temperature difference between the in- and outgoing cooling water does not exceed one degree or so, this gradient may be neglected for preparative separations. More powerful axial gradients, however, are developed at the upper and lower ends of the column. The temperature distribution at the upper end (Fig. 13) shows that already at a depth of 6–7 cm the end effect is negligible. These longitudinal gradients are probably not more significant than the radial gradients.

In columns without cooling jackets and with central cooling tubes of type I (*C*5, Fig. 4) the maximum allowable effect is determined by the local temperature increase near the nylon net. When loading is too high the heat generated is not transported away fast enough so that the solution finally boils and the column is spoiled. Already before this limit is reached the influence of the axial gradients on zone spreading in this region becomes very unfavorable. Cooling tubes of type II (*C*'5, Fig. 4) make possible more effective heat transport.

Radial temperature distribution in cylindrical starch and glass bead columns without central cooling may also be described by equation (11). Both curves in Fig. 14 are constructed with the same value of $K/\varkappa$ (3200) while the curves for the glass bead column (Fig. 15) are constructed with different $K/\varkappa$ (1930 and 1730). From the k values for cellulose and starch (1.45 and 4.12 respectively) and the conductivity of the electrolyte solutions at 15°C (3.6×10^{-3} ohm^{-1} cm^{-1}) equation (12) gives λ_c of 1.9×10^{-3} and 2.2×10^{-3} cal degree C^{-1} cm^{-1} sec^{-1} for cellulose and starch, respectively.

The heat conduction coefficient is constant for the Q values in question in preparative electrophoresis. In glass bead columns, however, λ_c begins to vary already at lower Q values. λ_c increases from 2.65×10^{-3} at 190 mA to 3.0×10^{-3} cal degree C^{-1}

cm^{-1} sec^{-1} at 275 mA. Apparently the conditions necessary for equation (2) to be valid are not even approximately met. Although equation (11) gives a good approximation of the radial temperature distribution at a given K or λ_c value determined for each Q, a considerable amount of heat must be transported through the column bed as a result of liquid flow. The variation in λ_c (or K) may therefore be regarded as a measure of the poor liquid stabilization against convection. That glass bead columns of this type are poorly suited for zone electrophoresis is also demonstrated by observation of stationary or migrating zones of non-adsorbing dyes, which rapidly spread in the column.

The water content in cellulose, starch and glass bead columns is ca. 85, 33 and 37 per cent per volume, respectively, and is apparently reflected in the k-values. From these values it is clear that cellulose, if other complications do not arise, should be preferred as stabilizer for large scale zone electrophoresis.

Electrophoretic separations

The current strength used for the separation of 4 g glutamic acid and 20 g glycine (Fig. 10), 500 mA, corresponds to a maximum radial temperature difference ($T_m - T_0$) of approximately 8.5° according to equation (7) ($\lambda_c = 1.9 \times 10^{-3}$ and $\lambda_g = 2.4 \times 10^{-3}$ cal degree C^{-1} cm^{-1} sec^{-1}). Without central cooling but with the same sectional area, however, half the current strength (according to equation (11a) approx. 260 mA) would yield the same maximum temperature difference. Already when the current strength exceeds 300 mA, the heat generated in a column of these dimensions without central cooling (with the same buffer) becomes too extensive to allow temperature equilibration. If zone spreading during filtration and electrophoresis, with the same maximum radial temperature difference, were the same in columns of the two types it would be possible to double the rate of separation by using central cooling. The different shapes of the temperature "profiles" and the disturbing influence of the central cooling tube on packing make comparison difficult, but according to our limited experience zone spreading during filtration and electrophoretic migration, given ideal loading conditions, seems to be approximately the same for both types of column.

Although the fractionation of aspartic and glutamic acid is not in itself of especial interest the large scale electrophoretic separation of these substances is of methodological interest. At pH 4.9 both amino acids migrate in the same direction. Consden *et al.* [6] found that the mobilities of aspartic and glutamic acid in 0.25 molar sodium phosphate buffer of pH 6.5 (the most favorable pH for the separation) were 1.92×10^{-4} and 1.73×10^{-4} cm^2 $volt^{-1}$ sec^{-1} in agar gel. The rather small difference in relative mobility makes this separation considerably more difficult than the former.

DNP-serine and DNP-aspartic acid have been included in small quantities to control electrophoresis and the compensating liquid counter flow. Aspartic acid and glutamic acid were completely separated after 74 hours (Fig. 11). The sharpness of the DNP-serine zone is due to the field strength gradient at the posterior part of the glutamic acid zone. A conductivity gradient but no significant pH changes could be demonstrated in the effluent within this region of the electropherogram. The sharp posterior regions of the glutamic acid and DNP-serine zones clearly show that the cooling tube, in spite of its bulky form at the bottom of the column does not cause serious disturbance. On the other hand, the spreading of the DNP-aspartic acid zone occurred at an approximately constant rate throughout the course of the

electrophoresis. The main cause of the zone spreading is not diffusion or convection caused by unstable density gradients but unequal liquid and material transport in filtration and electrophoresis which are dependent upon the heterogeneous structure of the cellulose. Viscosity is lower in the regions of relatively high temperature than at the walls. Hydrodynamic compensation can therefore be assumed to cause forced convection and thus increase the zone spreading. At low rates of flow, however, this effect is not too serious under the experimental conditions employed here.

In two filtration experiments 50 ml of solution containing 10 mg of DNP-aspartic acid was passed through a cellulose bed 1 meter in length; the majority of the DNP-aspartic acid (approximately 90 %) was contained in 200–250 ml of the eluate. Considering that migration of DNP-aspartic acid by electrophoresis and filtration is approximately 2.4 meters (i.e. 2.3 column lengths) the degree of spreading cannot be considered excessive. Instead it may be concluded that a lower current strength would not improve separation if the same separation capacity were to be retained. This would imply a wider starting zone. If the separation capacity is to be increased, column *B* must first be more effectively cooled. The ionic strength may also be lowered or the load raised since the ionic strength of the original solution in the demonstration experiment was considerably lower than that of the surrounding buffer.

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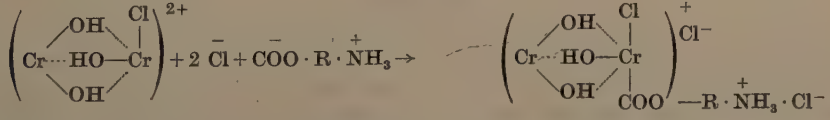
Approximation of the proportion of uni- and multi-point binding of basic chromium chlorides by collagen

By K. H. Gustavson

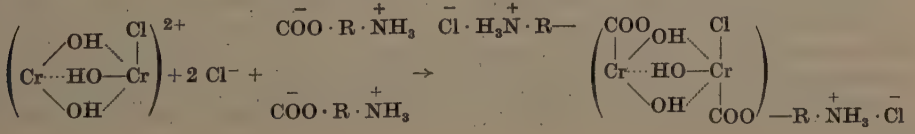
With 1 figure in the text

On reacting dilute solutions of basic chromium compounds, containing only electropositive chromium complexes, with the hide protein, collagen, the polyvalent cationic complexes are attracted to the negatively charged carboxyl ions of the collagen. The primary reaction of the discharge of the oppositely charged components is followed by the penetration of the carboxyl groups into the chromium complex, a covalent–coordinate linking of the carboxyl groups to the chromium atoms being formed (1). This concept of the fixation of cationic chromium compounds to the protein appears to be firmly established (1). Further, the high degree of stabilization of collagen (tanning) resulting from this reaction, in regard to the degree of hydrothermal stability and of the resistance to proteinases, has been accounted for by the formation of stable crosslinks between adjacent polypeptide chains of collagen by the multipointly fixed chromium complex (*intermolecular* crosslinking) (2). Such crosslinking which results in an increase of the mean molecular weight of soluble proteins, e.g. gelatin, has been experimentally proved (3). Numerous findings as to the behaviour of collagen are consistent with this view also (4). The mechanism of the fixation of a complex of the type $(Cr_2(OH)_2)^{4+}$ by the carboxyl groups of collagen which are interspaced in the helical structure is indeed a very complicated problem, since we do not know the distance between adjacent carboxyl ions in the collagen structure and the number of such groups which are close enough to be bridged by a complex of the size of the Cr–O–Cr complex of some 5 Å extension. It would appear likely that the main part of the binding of the polyvalent chromic cations occurs by one carboxyl ion, the surplus electropositive charge of the fixed chromium complex being compensated by Cl ions. Only a small part of the carboxyls would be expected to be located so favourably sterically as to allow a bipoint fixation. Then, the main reaction would proceed according to the schematic equation:

(1) 

where $\overset{-}{COO} \cdot R \cdot \overset{+}{NH_3}$ denotes the protein.

Only a minor part of the chromium complexes should then react as follows:

(2) 

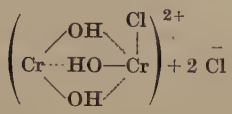
This important issue of the mechanism of chrome tanning was first realized some few years ago (5). The main evidence for the concept that the overwhelming part of the chrome fixation is due to the unipoint fixation of the polyvalent cation by the collagen was supplied by data from the reaction of dilute solutions of gelatin with basic chromium chlorides and sulfates (4, 5). After complete interaction of the chromium salt with the gelatin, that part of the chromium complexes which had not irreversibly combined with the gelatin was removed by treating the chrome–gelatin solution with cation exchangers of the sulphonic acid type. The amount of chromium in combination with the gelatin present in the filtrate from the ion exchange could then be estimated (5). It was found that the maximum uptake of chromium by the gelatin from solutions of basic chromium chlorides with composition corresponding to the empirical formula $Cr_2(OH)_2Cl_4$, was of the order of 14 % Cr_2O_3 of the weight of the gelatin, whereas the maximum chromium fixation for a tetrapoint fixation would be 3.8 % Cr_2O_3 and for a bipoint 7.6 %. The value of 14 % Cr_2O_3 found indicates an unipoint fixation to about 90 %, and a bipoint fixation of only 10 % (5). Moreover, the results of additional experiments by other means of attack were in harmony with the postulated view (4, 6).

In the present paper the results of an attempt of approximating the proportion of the uni- and multi-point (bi-point) types of reaction are presented. It is evident from equation (1) that the polyvalent chromium complex which only reacts with one carboxyl ion of the protein should contain its Cl groups in three different types of attachment: (i) Cl ions associated with the electropositive groups of collagen, (ii) Cl ions balanced by the electropositive charge of the fixed chromium complex, and (iii) the Cl groups directly attached to chromium in the complex. By a quantitative differentiation of these three types of combination, an estimate of the number of carboxyl ions reacting with *one* chromium complex, by means of *one* carboxyl group should be possible. Hence by difference, the extent of the reactions involving two or more carboxyl ions per chromium complex would be obtained. It should at the outset be pointed out that the high degree of stability of the complexly held sulphate groups (*sulphato*) compared to the chloride groups in basic chromium complexes (7) would make the former more promising as a reactant for this purpose. However, the basic chlorides offer certain distinct advantages over the corresponding sulphates by the greater simplicity of composition, their uniformity of electrical charge, being positively charged entirely, and the simple mode of the binding of the anion. The first paper of the series is therefore devoted to the basic chromium chlorides.

Experimental

A solution of basic chromium chloride, its composition corresponding to the empirical formula $Cr_2(OH)_2Cl_4 \cdot 2\,NaCl$, was prepared by adding one mole of NaOH in 2 molar solution to one mole of an AR grade of $(CrCl_2(OH_2)_4)\,Cl \cdot 2H_2O$, in a solution containing 2 moles Cr/liter. It was applied at a concentration of 1.2 equiv. Cr/l; the solution having been aged for 2 months. By the ion exchange technique, according to methods previously described (8), the sulfonic acid resin Dowex-50, operated in the Na form, showed 100 % cationic chromium. The total amount of Cl was contained in the filtrate. Hence, no chloro groups were present in the chromium complexes. By operating the anion exchange resin, Amberlite IRA 400, in the nitrate cycle (9), no anionic chromium was found and the Cl titration of the filtrate indicated

that at the most only 0.02 equiv. Cl were held per equiv. Cr in the chromium complex. According to results from diffusion experiments, the cationic complexes contain on the average 2 atoms Cr in the complex (10). From the data of the composition of the chromed collagen, it is indicated that in the present instance the average composition of the Cr compounds fixed by collagen corresponds to the empirical formula $Cr_2(OH)_3Cl_3$. The entrance of one Cl group into the chromium complex fixed by collagen is indicated by the data presented. Hence the probable structure of the compound reacting with collagen would be:



In order to ensure complete and uniform reaction of the chromium compounds with the hide protein, finely divided hide powder was selected as the substrate. It contained 18.0 % nitrogen. The ash content was less than 0.05 %. The amide content of this hide powder, which had been alkali-treated in its preparation, was 0.28 mequiv. NH_2 per g protein (11). Since the total content of carboxyl groups of collagen is 1.26 mequiv. per g protein (12), the amount of free carboxyls was 0.98 mequiv. Its acid-binding capacity on equilibration with 0.1*N* solution of HCl, made 0.75*M* in NaCl, at pH 1.4 was 0.94 mequiv. HCl per g collagen. The alkali-binding capacity (NaOH) at an equilibrium pH of 12.2 was 0.61 mequiv. per g collagen.

To 25 g of hide powder, previously hydrated in 50 ml of water, 250 ml of the solution of the 67 % acid chromium chloride, 1.2 equiv. Cr per liter, were added. The final concentration of the bath was thus 1.0 equiv. Cr/l. After intermittent shaking for 120 hours, the chromed hide powder was freed from the extraneous solution, which had a pH value of 2.85, by suction filtering. After a light washing, the "sucked-off" chromed hide powder was further treated for 120 hours in 200 ml of a fresh solution of the same basic chloride; containing 1 equiv. Cr/1, with final pH value of 2.9. The final product was freed from the chrome solution by suction filtering, washed quickly under suction, rotated for 10 minutes in one liter of water, and finally sucked free from solution. It contained 31.6 % of dry substance. A portion was dried at 105° and analyzed. The analytical data are given in Table 1.

By treating the chrome-collagen compound obtained by the irreversible fixation of the basic chromium salt by collagen in water maintained at a constant pH of 8.2, the anions held by the basic protein groups and those electrostatically balanced by the fixed chromium complexes, which carry an excess of positive electric charge, are removed (13). By equilibration of the chromed hide powder at pH 5, by far the greatest part of the protein-bound acid (anions held by the basic protein groups) is removed (14). The main part of the anions electrostatically associated with the fixed chromium complexes is apparently not affected by this treatment. Buffer solutions usually contain anions, such as acetate and phosphate, which are effective complexers of chromium (13, 15). Hence, coordinated groups in the chromium complexes are more or less removed by such buffers. Therefore buffer solutions cannot be used for the estimation of the distribution of the chloride and sulphate groups which possess comparatively low complexing power for chromium (7, 15).

Evidently there are three forms of attachment of the anions to the chromed collagen (5). These forms are in equilibrium with each other. A disturbance of one of them will influence the others and change the distribution of the various types of

association of the anions. This is particularly the case with the basic chromium chlorides. The Cl group has a very much lower affinity for the cationic protein groups and especially for the chromium atom than the sulphate anion of the corresponding basic chromium sulphates in solutions of comparative concentration of chromium and with the same neutral salt content.

The following procedures were applied for an approximate evaluation of the different forms of association of the chloride groups in the chrome-collagen compound obtained by tanning hide powder with the basic chromium chloride. Portions of 6.33 g of the moist chromed hide powder (freed from extraneous matter by suction filtering), equal to 2.00 g of collagen, were suspended in 800 ml of water for 16 hours, occasionally shaken. The acid diffusing out from the substrate into the water was titrated with 0.1*N* solution of NaOH to pH 5 (methyl red). The containers were then shaken for 15 minutes. The solutions were then restored to pH 5 by the addition of the necessary amount of alkali. This procedure was repeated every 15 minutes during the first two hours of shaking and then every half hour, with a total running time of 6 hours. The treated specimens of the chromed hide powder, with its protein bound acid removed, was then sucked free from the solution, lightly washed, dried and analyzed. Corresponding series were run with the original untreated hide powder, which had been equilibrated with a solution of 0.1*N* HCl at pH 1.3, containing 5 vol. % NaCl as a swelling depressing agent. After 6 hours' shaking the treated hide powder was separated from the solution by suction filtering and washed quickly with a 5 % NaCl solution, in order to remove the main part of the free acid in the interstices of the hide powder. From the acid content of the original and the final solutions, including the wash water, the amount of acid fixed by the hide powder was obtained by difference. The acid binding capacity of the collagen was evidently satisfied, since 0.98 mequiv. of HCl were removed by 1 g collagen; the theoretical value of the maximum binding capacity of collagen for HCl under the conditions given being 0.90–0.94 mequiv. This acid-saturated hide powder was treated in the same way as the chromed hide powder simultaneously. Complete removal of the acid from the hide powder was effected in 5 hours' treatment at pH 5 by the diffusion–neutralization technique (cf. (14)).

All the chloride groups except those bound in the complex, were removed by shaking portions of the moist chromed hide powder, equal to 2.00 g of collagen, in 100 ml portions of 4 % solution of pyridine for one hour. The quantity of acid removed by the pyridine was determined by titration of the used solution and the washings with 0.1*N* NaOH solution to pH 8.2 (phenolphthalein) (16). The treated stock was freed from the solution by suction filtering, washed and finally dried at 110°C. This temperature was used in order to remove any remaining pyridine from the stock (13). Simultaneously the same quantity of chromed hide powder was equilibrated at pH 8.2 (phenolphthalein), employing the diffusion–neutralization method described, the time of treatment being prolonged to 8 hours, however.

The rate of the removal of the acid by equilibration of the chromed hide powder in water at pH 5 and pH 8.2 by gradual addition of alkali is shown graphically in Fig. 1.

From the amount of alkali consumed in the various treatments on diffusion–neutralization and by the pyridine solution used for the treatment, the amounts of the various types of Cl groups are obtained. From the analysis of the dried chromed specimens, an approximation of the composition of the original chromed hide powder as to the distribution of Cl groups and of chromium in uni- and multi-point binding

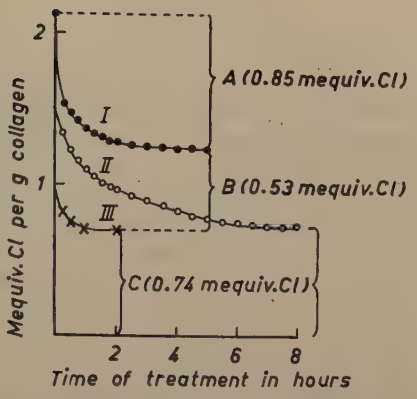


Fig. 1. The rate of removal of Cl ions from chromed collagen, with its binding capacity for chromium complexes of basic chlorides completely satisfied, on equilibrating the substrate in aqueous system at: I, pH 5.0; II, pH 8.2; III, pH 8.2 (pyridine solution). The amounts of the various forms of Cl groups in the chrome-collagen compound are indicated by the braces as: *A*, Protein-bound Cl; *B*, Cl as Gegen-ion of unipoint fixed chromium; *C*, Cl held in the chromium complex (chloro groups).

can be inferred. Table 1 contains the data on the Cr content and the Cl content of the original chromed hide powder and the variously treated specimens of the chromed hide powder. The % acidity gives the equivs. of Cl in the substrate as a percentage of the equivs. of Cr in the substrate. Table 2 shows the distribution of the chromium complexes and the chloride groups in the original chromed hide powder obtained from the data in Table 1. In Table 2 the the corresponding figures obtained by the analysis of the solutions for the diffused or removed chloride are also included (*indirect* estimation). The figures are given in mequiv. Cr or Cl per g collagen.

Table 1. Composition of the chromium-chloride-tanned hide powder.

No.	State	Cr	Total Cl	% acidity of chrome-collagen compounds
a	Original	4.10	2.12	52
b	Pyridine-treated	4.10	0.71	17
c	Equilibrated at pH 5	4.10	1.26	31
d	Equilibrated at pH 8.2	4.10	0.73	18

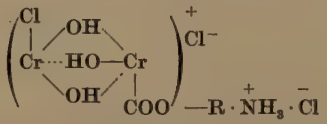
Table 2. Distribution of Cr complexes and Cl groups in the chrome-collagen compounds.

State of binding	Mode of calculation	Directly obtained by analysis (from Table 1)	Indirectly obtained from solutions (data in Fig. 1)
Cl, associated with the cationic protein groups	(a–c)	0.86	0.85
Cl, complexed with Cr (chloro groups)	b	0.71	0.74 (by difference)
Cl, electrostatically held by the unipointly fixed Cr complexes	(c–b)	0.55	0.53

Discussion

The data on the composition of the chromed hide powder indicate that from the protolyzed solution of the 67 % acid chromium chloride the protein has removed a compound or compounds with an average composition corresponding to the empirical formula $Cr_2(OH)_3Cl_3$, i.e. the 50 % acid compound. The additional small amount of Cl groups, corresponding to 2 % acidity, present in the tanned hide powder has probably been introduced by the reaction of the protons and Cl anions in the protolyzed system with collagen. The protons compete with the cationic chromium complexes for the carboxyl ions of collagen, and its anions with the Cl ions of the chromium chloride for the cationic protein groups, in order to maintain the electroneutrality of the system which has been disturbed by the removal of the cations from the solution (1). Moreover, variations in the composition of the chloro-chromium complexes as to their content of Cl groups and the aggregation of the fixed chromium complexes *in situ* may be contributory factors. In the present instance these slight variations do not affect the deductions of the results, nor the final conclusions to be drawn.

It is remarkable that while the chromium complexes present in the solution of the 67 % acid chloride did not contain any complexly bound Cl groups, the chromium complexes fixed by collagen contained on the average one Cl group in the binuclear complex. Thus, the average composition of the chromium complexes fixed by collagen corresponds to the schematic structure: $(Cr_2(OH)_3Cl)\prec$. The unifunctionally reacting complexes should then be present in the chrome-tanned collagen as structures of the type:



It should also be noted that stabilization of the collagen lattice, i.e. crosslinking of the polypeptide chains, is obtained on reacting collagen, which has been equilibrated at pH 3 with solution of hydrochloric acid in the presence of swelling–depressing sodium chloride, with the solution of the basic chromium chloride employed (with a pH value of 3 also). This fact of the identical pH values of the components of the system excludes the occurrence of any appreciable increase of the size of the fixed chromium complexes by secondary reactions *in situ*, due to and involving disturbance of the protolytic equilibrium by the binding of protons by the collagen. Such a secondary aggregation of the fixed chromium compounds is indicated to occur to some extent on reacting isoelectric collagen (pH 5) with solutions of chromium salts of much higher H ion concentration (pH 2.5–3.5).

If the total amount of chromium fixed by collagen should be present in unipoint attachment, the quantity of Cl groups held outside the chrome complexes should be $4.1/6 = 0.68$ mg equiv. per g collagen. According to the data from the direct analysis of the stock, this type of Cl groups amounts to 0.55 mequiv. per g collagen. Accordingly, (0.55/0.68) 100, or 81 % of the total chromium (expressed as equiv.), is present in the form of unipointly bound complexes. The indirect method gives the corresponding figure of 78 %.

Concerning the reactivity of the carboxyl ions of collagen, the following points are pertinent. Approximately 0.7 mequiv. carboxyl ions per g collagen is discharged

by the protons on equilibration with a solution of hydrochloric acid at pH 3, made 0.3 molar in NaCl. In view of the strong complexing power of the carboxyl ion for chromium and the mass action effect, the principal reaction would be expected to occur between the cationic chromium complexes and the collagen. Actually, on treatment of collagen equilibrated with hydrochloric acid at pH 3 with solutions of basic chromium chloride of the same pH, displacement of protons by the cationic chromium complexes takes place (17). By the discharge of the carboxyl ions by the chromium cations, resulting in the transformation of the ionic linkage into the co-ordinate–covalent type of linkage, the equilibrium between the charged and uncharged carboxyl groups is disturbed. Accordingly the chrome fixation will proceed. Hence, the amount of 0.7 mequiv. carboxyl ions per g collagen, which is estimated to be discharged by the cations in the present instance, is probably a minimum value.

For an evaluation of the number of carboxyl ions taking part in the chrome fixation, an estimate of the amount of free carboxyl ions in the chromed collagen is required. Its acid-binding capacity is an important factor. This is a very difficult determination, since the acid which has to be added for this approximation is not restricted in its reaction to the ionic protein groups present. The chromium complexes fixed in the collagen structure also interact with the acid. However, by keeping the time of the treatment of the chromed collagen with the acid as brief as possible, this complication can be largely eliminated. With the time of interaction limited to half an hour, the finely divided chromed hide powder and the intact hide powder used as blank will practically attain the maximum fixation of acid upon vigorous shaking, with only a slight removal of chromium by the acid.

In a separate experiment, the amount of chromium removed from the chromed hide powder in the acid treatment was determined, correcting for the acid used, assuming $CrCl_3$ to be formed. By shaking an amount of the moist tanned hide powder, which had been aged 2 weeks, equal to 1.00 g collagen in 25.0 ml 0.1*N* HCl solution for 30 minutes, an amount of acid equal to 0.08 mequiv. HCl was fixed by 1 g collagen (corrected value). The intact hide powder concurrently removed 0.90 mequiv. acid per g collagen. Since an amount of 0.85 mequiv. Cl was present in the chromed collagen in the form of protein-bound acid, the total amount of cationic protein groups was 0.93 mequiv., which is the value of the acid-binding capacity of collagen. The number of free carboxyl ions in the chromed collagen which are able to react with cations is thus 0.08 mequiv. per g collagen. The intact hide powder contained 0.98 mequiv. carboxyl groups per g collagen.

The approximation of the number of carboxyl ions involved is in reasonable agreement with the analytical data if it is assumed that 0.75 mequiv. of the total number of 0.98 mequiv. potential carboxyl ions per g collagen are able to react with the chromium complexes. According to the figures for the distribution of the unipoint and multipoint fixation of chromium by collagen, 80 % of the reacting carboxyls, or 0.60 mequiv. per g collagen, should then have fixed *one* polyvalent binuclear chromium complex by *one* carboxyl group. That would correspond to an amount of Cr_2O_3 equal to 0.6×152 mg, or 9.1 %, on the basis of collagen. The remaining 0.15 mequiv. COO^- groups reacting *bifunctionally* should then fix 0.15×76 mg Cr_2O_3 or 1.2 % Cr_2O_3 on the weight of collagen. Accordingly, the total chrome fixation should amount to 10.3 % Cr_2O_3, on the weight basis of collagen. The directly determined figure was 10.4 %. Recalculating the amounts of chromium fixed by collagen in the two forms of binding, the figure for the bipointly fixed chromium will be 12 % of the total amount of chromium fixed. The rest, or 88 % of the total chrome fixation,

is accounted for by the main type of attachment of chromium to collagen, the unipoint fixation of the polyvalent binuclear chromium complexes.

In an earlier investigation of the reactivity of basic chromium chlorides with proteins by an entirely different mode of approach (5) than the present one, i.e., by the study of the interaction of the chromium compound employed with gelatin in dilute aqueous solution (0.8 %), an approximation of the proportion of the *uni-* and *multi*-point binding of this compound by the protein showed that the unifunctional fixation was of the order of 90 % of the total amount of chromium reacting and fixed by the protein. Great importance is attached to the fact that the corresponding values of the distribution of the more stable sulphate groups in chromium sulfate-tanned collagen, which had been treated and analyzed in the manner described in this paper, show the same trend and are of the same order of magnitude. The only disturbing element in the present instance is the entrance of one Cl group into the binuclear chromium complexes on their fixation by collagen from a solution which is devoid of chloro-chromium complexes. However, in concentrated solutions of basic chromium chlorides of the same composition as the present one investigated, corresponding to $Cr_2(OH)_2Cl_4 \cdot 2\,NaCl$, chloro groups are present (18). A possible explanation of the existence of chloro groups in leather tanned by means of solutions of basic chromium chlorides free from complexely bound Cl groups should be that in the interstices of the hide powder surface layers of extremely high chrome concentration may be built up during the reaction and the subsequent diffusion. Such a concentration increase would favour the formation of chloro-chromium complexes. The setting-up of very high concentration gradients in the surface layers of gelatin gels is indicated to occur on the diffusion of certain dyestuffs into the gel, densitometrically determined, according to the recent researches of Jelley and Pontius (19). Actually, the concentration of dyestuff in the surface layers may reach values about 200 times greater than the concentration of dyestuff in the external solution.

Further it has been shown that in the fixation of formaldehyde by collagen (20) only about one eighth of the formaldehyde fixed by the basic groups of collagen contributes to the crosslinking (bifunctional reaction), while the rest is unipointly attached as methylol groups. These findings were obtained by the study of the stress–strain curves of heat-contracted formaldehyde–collagen (tendon), which relationship is a function of the segmental molecular weight, i.e. the weight of the segment of the protein chain between adjacent interchain crosslinks (methylene bridges). The application of the same method to hydrothermally contracted, lightly chrome-tanned tendon shows the same trend (21). Further, it is evident from this type of experiments that the bipoint fixation, i.e. the crosslinking reaction, dominates in the early stage of the reaction (20) and consequently that the fixation of the agent in the later stages of the reaction mainly occurs by unipoint attachment. Unpublished data show this trend for the reaction of collagen with solutions of basic chromium salts also. However, on account of the presence of large quantities of free reactive groups in the chromed collagen of low chrome content, as obtained in the initial fixation, the approximation of the order of the uni-multi-functional reactivity is very much more difficult than in systems with attained equilibrium and with practically no reactive protein groups left intact.

From the changes of the thermodynamic constants, imparted to collagen by its fixation of basic chromium salts, Weir (22) concludes that the crosslinking of the protein chains by the chromium complexes reaches its maximum at such comparatively low content of fixed chromium as 1 % Cr_2O_3 on the weight of collagen. The approxi-

mation of the amount of bipointly fixed chromium chlorides found in the present investigation is of the same order of magnitude as Weir's value.

The figures given for the order of the bipoint reaction, i.e. crosslinking, which will stabilize the collagen lattice are actually maximum values because the crosslinking should be expected to be of two entirely different types from the point of view of stabilization of the molecular chains and protofibrils of collagen. Bridges may be formed by the tanning agent between groups in the same polypeptide chain, the *intramolecular* type of crosslinking, which is not expected to increase the hydrothermal stability of the protein to the same extent as the second form, the *intermolecular* type of crosslinking, which will rivet together adjacent chains of the polypeptides. The relative importance of these two types of bridging the protein groups cannot be evaluated at the present lack of knowledge of the steric conditions in the collagen lattice. This important issue has to be postponed until information on the distances between the reactive groups in the protein structure becomes available.

Summary

The order of magnitude of the unipoint fixation by collagen of polyvalent binuclear complexes from aqueous solutions of basic chromium chlorides has been estimated by determining the distribution of the chloride groups in the resulting chrome-collagen compounds. Their protolyzable chloride is present in three forms. Cl groups attached to (i) the cationic protein groups, (ii) the chromium complexes fixed by collagen, which carry surplus positive charge, and (iii) the chromium in the complex (chloro groups).

It is inferred from the data obtained that on complete inactivation of the carboxyl ions of collagen by the cationic chromium complexes fixed from the 67 % acid chromium chloride, with composition corresponding to the empirical formula $Cr_2(OH)_2Cl_4 \cdot 2\,NaCl$, about 90 % of the total fixation of chromium is due to the discharge of one carboxyl ion of collagen by one polyvalent binuclear chromium complex. The remaining 10 % of the chrome uptake by collagen is accounted for by multipoint fixation. The stabilization of the collagen structure by crosslinking is assumed to result from the fixation of the chromium complexes which are reacting multifunctionally with at least two protein chains.

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